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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/63, 15/82, C12Q 1/68, C12N 5/10	A2	(11) International Publication Number: WO 00/52179 (43) International Publication Date: 8 September 2000 (08.09.00)
(21) International Application Number: PCT/US00/05728 (22) International Filing Date: 3 March 2000 (03.03.00) (30) Priority Data: 60/122,513 3 March 1999 (03.03.99) US 60/154,605 17 September 1999 (17.09.99) US (71) Applicant: GENELABS TECHNOLOGIES, INC. [US/US]; 505 Penobscot Drive, Redwood City, CA 94063 (US). (72) Inventors: LIM, Moon, Young; 10 Lido Circle, Redwood City, CA 94065 (US). EDWARDS, Cynthia, A.; 16965 Pine Way, Morgan Hill, CA 95037 (US). FRY, Kirk, E.; 2604 Ross Road, Palo Alto, CA 94303 (US). BRUCE, Thomas, W.; 6880 Shearwaters Drive, Carlsbad, CA 92009 (US). STARR, Douglas, B.; 1197 Bonita Avenue, Mountain View, CA 94040 (US). LAURANCE, Megan, E.; 1020 Shotwell Street, San Francisco, CA 94110 (US). KWOK, Yan; 995-1 Belmont Terrace, Sunnyvale, CA 94086 (US). (74) Agents: JUDGE, Linda, R. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: DNA BINDING COMPOUND-MEDIATED MOLECULAR SWITCH SYSTEM		
(57) Abstract <p>The present invention provides molecular switch system methods and compositions for use in regulatable gene expression. The system includes a nucleic acid construct which has a DNA response sequence for a transcriptional regulatory protein operably linked to a promoter, a compound binding sequence in the vicinity of the DNA response sequence, a transgene under the control of the promoter, and a DNA binding compound. In some cases, the molecular switch system further includes a nucleic acid sequence encoding a transcriptional regulatory protein operably linked to a second promoter. The invention further provides a method for screening compounds for the ability to function in the molecular switch system and thereby regulate gene expression.</p>		

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DNA BINDING COMPOUND-MEDIATED MOLECULAR SWITCH SYSTEM

Field Of The Invention

5 The present invention relates to methods for the regulated expression of a gene using cells which comprise a molecular switch, including a transcriptional regulatory protein, a DNA response site for the transcriptional regulatory protein, and a compound binding sequence in the vicinity of the DNA response site, such that sequence-dependent binding of a compound to the compound binding sequence modulates expression of a gene operably linked thereto.

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Background Of The Invention

Regulated gene expression has utility in a variety of applications including the expression of recombinant proteins, modified production of various metabolites, functional studies in cell-based assays and *in vivo* in transgenic animals, in gene therapy vectors, and in plant expression vectors for controlled transgene expression.

Gene therapy is a fast evolving area of medical and clinical research. Gene therapy encompasses gene correction therapy and transfer of therapeutic genes and is being applied for treatment of cancer, infectious diseases, monogenic diseases, multigenic diseases, and acquired diseases.

There are an increasing number of anecdotal cases of efficacy in the use of gene therapy for the treatment of monogenic diseases, early stage tumors, and cardiovascular disease (Blaese, *et al.*, 1995; Wingo, *et al.*, 1998; Dzau, *et al.*, 1998; Isner, *et al.*, 1998). However, all of the currently utilized methods of gene transfer typically demonstrate low transfer efficiency and expression rates. As the technology is improved and high efficiency gene transfer and expression is achieved, the ability to regulate such expression on both a temporal and spatial level becomes increasingly important.

In addition, the development of plants having desired traits such as improved yield; disease resistance to fungal, bacterial, viral and other pathogens; insect resistance; improved fruit ripening characteristics; cold temperature and dehydration tolerance; increased salt and drought tolerance; improved food quality (*i.e.*, nutritional content) and improved appearance has been the focus of agribusiness for many years. At present, the regulated expression of transgenes in plants with optimal expression of target genes in manner that does not result in harm to the plant is the focus of extensive research.

Attempts to control gene activity have been made using various inducible eukaryotic promoters, such as those responsive to heavy metal ions, heat shock or hormones. In most cases, the effect of exogenous inducers is pleiotropic, in that it induces the expression of endogenous cellular genes in addition to the target transgene. Second, many promoter systems exhibit high levels of basal activity in the non-induced state, *i.e.*, endogenous activators often interfere with regulation of transgene expression.

Several systems for regulatable expression of genes ("gene switch" systems) have been reported in the literature. Such systems are based on modifying the activity of synthetic regulatory proteins, which bind to double stranded DNA and control the activity of a promoter for a given gene, by the use of exogenous inducers (compounds) that specifically interact with a particular synthetic regulatory protein.

In systems where an inducer interacts with a regulatory protein, the regulatory protein dictates the selection of inducer. So, the ability to choose an inducer with better pharmacological properties are limited by the selection of regulatory protein.

Methods for screening and constructing molecules, which have properties of sequence specific DNA binding and displacement of protein that is bound at flanking or

adjacent sites on a DNA sequence, have been reported in co-owned U.S. Pat. Nos. 5,306,619, 5,693,463, 5,716,780, 5,726,014, 5,744,131, 5,738,990, 5,578,444, 5,869,241.

Using such methods, several classes of small molecules that interact with double-stranded DNA have been identified, and shown to preferentially recognize specific nucleotide sequences.

A need exists for the development of systems for regulatable gene expression which are controlled, inducible by compounds targeted to polynucleotides, and characterized by low toxicity and favorable pharmacokinetic properties.

Summary Of The Invention

The invention provides a molecular switch which employs a natural, engineered or synthetic DNA binding transcriptional regulatory protein and a compound (inducer) that interacts with double stranded DNA in the vicinity of the transcriptional regulatory protein binding site or DNA response element.

The binding of the compound to DNA affects the binding of the transcriptional regulatory protein to its DNA response element, thereby modifying the expression of a gene operably linked to the DNA response element.

More particularly, the invention provides a molecular switch which includes a first nucleic acid construct that has a DNA response sequence for a transcriptional regulatory protein operably linked to a first promoter; a compound binding sequence in the vicinity of the DNA response sequence for binding to a DNA binding compound; a transgene under the control of the first promoter; and a DNA binding compound.

In some cases, the molecular switch includes an engineered, non-native exogenous or synthetic transcriptional regulatory protein, by providing a second nucleic acid sequence having the coding sequence for a transcriptional regulatory protein operably linked to a second promoter.

The molecular switch may take the form of a single vector comprising one or more promoters, or may take the form of a two vector embodiment, wherein each vector comprises a promoter, which may be the same or different.

Promoters for use in the molecular switch may be compound inducible or constitutive promoters.

The molecular switch may provide from 1 to 12 compound binding sequences, wherein each compound binding sequence has from about 8 to 20 nucleotides.

The molecular switch may further provide from 1 to 12 tandem repeated transcriptional regulatory protein DNA response sequences.

The invention further includes a method of producing cells comprising a molecular switch for modulating gene expression, and cells produced by that method.

A method of screening DNA-binding compounds for the ability to regulate a molecular switch is also included in the invention and is based on: (i) identifying a DNA sequence to which a DNA binding compound is to bind; (ii) providing a nucleic acid construct having a DNA response sequence for a transcriptional regulatory protein and a compound binding sequence in the vicinity of the DNA response sequence; (iii) screening a plurality of candidate DNA binding compounds, by exposing each of the candidate compounds to the

nucleic acid construct and identifying DNA binding compounds having the ability to bind to the compound-binding sequence.

Brief Description Of The Figures

5 Figure 1 is a schematic illustration of a transcriptional regulatory protein/DNA binding compound-mediated molecular switch system, wherein a transcriptional regulatory factor (TF, consisting of a transcriptional activator or repressor domain and a compound-binding domain), which may be native to a cell or provided exogenously in a plasmid (pTF), interacts with a response element (RE) comprising a ligand binding site (LBS) and a
10 transcriptional regulatory factor binding site (TFBS). Components of the system include a transcription factor, a small molecule or ligand and a switchable promoter construct.

Figure 2A shows the consensus sequence of the *rrnB* P1 promoter UP element which has been previously described (Estrem *et al.*, 1998).

Figure 2B shows the sequence of nucleotides -66 to +50 of the *rrnB* P1 promoter.

15 Figure 3 depicts exemplary switchable promoter constructs engineered to have a compound, ligand or drug binding sequence near the *cis* element, with the transcriptional regulatory protein DNA response element indicated as bolded and uppercase, the introduced nucleic acid sequence for compound binding indicated in lowercase and potential compound binding sequences indicated as () or []. In such constructs, the compound binding sequence
20 may be introduced relative to the transcriptional regulatory protein DNA response element, in one or more locations including: (1) on either side, (2) on both sides, (3) upstream, (4) downstream, or (5) overlapping the DNA response element.

Figure 4A depicts various oligonucleotide constructs engineered to have a compound-binding sequence, indicated as () or [], in the vicinity of *rrnB* P1 promoter UP element.

25 Figure 4B depicts the effect of various concentrations of 21x on reporter expression in *E. coli* strains that carry *rrnB* P1 promoter constructs (the sequences for which are presented in Fig. 9A), fused to a *lacZ* reporter on the chromosome as a phage mono-lysogen, as indicated in the figure. Cells were incubated with or without 21x for 24 hrs and promoter activities assayed following treatment. Promoter activities are expressed as a percentage of
30 basal promoter activity. All samples were in triplicate, the error bars represent standard errors of the mean (SEM) for three separate experiments.

Figure 5 depicts the upper strand of various double-stranded oligonucleotides engineered to have a compound-binding sequence in the vicinity of a UL9 DNA response element, wherein the transcriptional regulatory protein DNA response element is indicated as
35 bolded and uppercase, introduced compound binding sites are indicated in lowercase and potential compound binding sites are indicated as () or [].

Figure 6 depicts the results of DNA binding studies with the modified UL9 DNA response sequences presented in Fig. 9A and ³²P labeled oligos, incubated with various concentrations of 21x. The modified sequences include "YK 202LX" (shown as diamonds, SEQ ID NO:18), "YK 202RX-A" (shown as squares, SEQ ID NO:19), and "YK 202RX" (shown as triangles, SEQ ID NO: 21).
40

Figure 7 depicts the upper strand of various double-stranded oligonucleotides engineered to have a drug-binding sequence overlapping an p50 NF-KB DNA response

element, with the transcriptional regulatory protein DNA response element indicated as bolded and uppercase, introduced drug binding sites indicated in lowercase and potential drug binding sites indicated as () or [].

Figure 8A depicts the results of DNA binding studies with the modified p50 NF-KB DNA response sequences of 21x. The modified sequences include "JF101" (shown as diamonds, SEQ ID NO:31), "JF102" (shown as squares, SEQ ID NO:32), and "JF103" (shown as triangles, SEQ ID NO:33).

Figure 8B depicts the results of DNA binding studies with the modified p50 NF-KB DNA response site, JF102 and ³²P labeled oligonucleotides, incubated with various concentrations of distamycin.

Figure 9 depicts the results of DNA binding studies with the modified LacR DNA response sequences (lacO) and ³²P labeled oligos, incubated with various concentrations of 21x. The modified sequences include the sequence presented as SEQ ID NO:34 (shown as squares) and the sequence presented as SEQ ID NO:35 (shown as diamonds).

Figure 10 depicts the results of DNA binding studies with a modified LacR DNA response sequence (SEQ ID NO:35) and ³²P labeled oligos, incubated with various concentrations of 21x (shown as diamonds) or IPTG (shown as squares).

Figure 11 depicts the effect of 21x on the activity of the chimeric activator ULVP on various promoter constructs driving firefly luciferase, transfected into MCF7 cells. Transfected cells were incubated with or without 21x for 48 hrs and promoter activities assayed at 48 hrs post-transfection. Promoter activities were normalized relative to the co-transfected internal control (pRL-NUL basal promoter) driving Renilla luciferase and expressed as a percentage of the untreated wild-type promoter construct.

Figure 12 depicts the effect of 21x on various cyclin D1 promoter derivatives driving firefly luciferase in pGL3 basic, transfected into MCF7 cells, as indicated on the Figure. Transfected cells were incubated with or without 21x for 48 hrs and promoter activities assayed at 48 hrs post-transfection. Promoter activities were normalized relative to the co-transfected internal control (pRL-NUL basal promoter) driving Renilla luciferase and expressed as a percentage of the untreated wild-type promoter construct. All samples were in triplicate, the error bars represent standard errors of the mean (SEM) for three separate experiments.

Figure 13 depicts the dosage-dependent effect of the DNA-binding compound GL046732 on the activity of engineered HBV core promoter constructs driving firefly luciferase in pGL3 basic, in HepG2 cells, where CpWT is the core promoter wild type construct (SEQ ID NO:51), CpTATARds1 (SEQ ID NO:55) and CpHNF3Rds1 (SEQ ID NO:58), have ds1 sequences placed adjacent and overlapping the TATA and proximal HNF3 site, respectively.

Figures 14 A and B depict the sequence of the pACT ULVP activator construct (SEQ ID NO:61).

Figures 15 A and B depict the sequence of the pACT ULKRAB repressor construct (SEQ ID NO:62).

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

As used herein, a nucleic acid may be double stranded, single stranded, or contain

portions of both double stranded or single stranded sequence. The depiction of a single strand also defines the sequence of the other strand and thus also includes the complement of the sequence.

5 As used herein, the term "recombinant nucleic acid" refers to a nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid.

10 A "heterologous nucleic acid construct" has a sequence portion which is not native to the cell in which it is expressed. Heterologous, with respect to a control sequence/coding sequence combination refers to a control sequence (*i.e.*, promoter or enhancer) together with a coding sequence or gene, that is not found together in nature, in other words, the promoter does not regulate the expression of the same gene in the heterologous nucleic acid construct and in nature. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell, by transfection, microinjection, electroporation, or the like. Such a heterologous nucleic acid construct may also be referred to herein as an "expression cassette".

15 As used herein, the term "vector" refers to a nucleic acid construct useful for transfer of the vector between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

20 As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a vector, and which forms an extrachromosomal self-replicating genetic element in many bacteria and some eukaryotes.

25 As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide, which may or may not include regions preceding and following the coding region. For example, 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons), may or may not be included in the DNA segment designated as the gene.

30 As used herein the term "transgene" refers to the portion of a heterologous nucleic acid construct, expression cassette or vector which comprises the coding sequence for a polypeptide, wherein the gene is associated with other components, *i.e.*, the promoter with which it is not normally associated in nature.

35 As used herein, the term "regulatable expression system", or "molecular switch system" includes the DNA response element (site or sequence) for a transcriptional regulatory protein, a promoter, a compound-binding sequence, and a DNA binding compound. In some cases, the "regulatable expression system", or "molecular switch system" further includes an exogenously provided transcriptional regulatory protein.

As used herein, the term "DNA response element" refers to the DNA binding site or sequence for a transcriptional regulatory protein, which may be the same as, overlapping, or adjacent to, a compound-binding sequence.

40 As used herein, the terms "compound binding sequence", "compound binding site", "ligand binding sequence", and "ligand binding site" are used interchangeably and refer to the portion of a DNA sequence with which a compound, ligand, or molecule interacts resulting in the modified binding of a transcriptional regulatory protein to its DNA binding

site (or DNA response element). In some cases the compound, ligand, or molecule may also be designated a compound or inducer. The "compound-binding sequence" or equivalent is in the vicinity of the DNA response element for transcriptional regulatory protein and may be adjacent (*i.e.*, flanking), overlapping, or the same as the DNA binding site for a transcriptional regulatory protein.

As used herein, the term "promoter" refers to a sequence of DNA that functions to direct transcription of a gene which is operably linked thereto. The promoter will generally be appropriate to the host cell in which the target gene is being expressed. The promoter may or may not include additional control sequences (also termed "transcriptional and translational regulatory sequences"), involved in expression of a given gene product. In general, transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. The promoter may be homologous or heterologous to the cell in which it is found.

As used herein, the terms "regulatable promoter", "inducible promoter" and "switchable promoter", are used interchangeably and refer to any promoter the activity of which is affected by a *cis* or *trans* acting factor.

As used herein, the terms "transcriptional regulatory protein", "transcriptional regulatory factor" and "transcription factor" may be used interchangeably with the term "DNA-binding protein" and refer to a cytoplasmic or nuclear protein that binds a DNA response element and thereby transcriptionally regulates the expression of an associated gene or genes. Transcriptional regulatory proteins generally bind directly to a DNA response element, however in some cases may bind indirectly to the another protein, which in turn binds to or is bound to the DNA response element.

As used herein, the term "transcriptional regulatory fusion protein" refers to a recombinant fusion protein consisting essentially of a DNA binding domain and a regulatory domain. The terms "chimeric protein" and "fusion protein" are used interchangeably herein, and refer to the transcriptional regulatory fusion proteins of the invention. It will be understood that in some cases a DNA binding protein may lack a regulatory domain and that the methods of the invention are also applicable to such transcriptional regulatory proteins.

Such a transcriptional regulatory protein may be (1) natural (native), (2) chimeric (chimera of the DNA-binding domain of a natural protein and the regulatory (activator or repressor) domain of a natural protein, (3) synthetic, having a novel DNA-binding domain designed by structural modeling, phage display screen, or other methods, and (4) may or may not take the form of a fusion protein.

As used herein, the terms "natural regulatory factor", "natural regulatory protein", "native regulatory factor", and "native regulatory protein" are used interchangeably and refer to transcriptional regulatory factors that are either broadly effective, tissue-specific, disease-specific or heterologous natural (native) factors. Such factors may be provided exogenously or may be endogenous to a particular tissue or cell type.

As used herein, the terms "synthetic regulatory factor", "synthetic regulatory protein" and "engineered regulatory factor", are used interchangeably and refer to factors that are non-native (not natural) to the host, and are provided exogenously to a cell.

As used herein, the term "operably linked" relative to a recombinant DNA construct or vector means a nucleotide component of the recombinant DNA construct or vector is in a functional relationship with another nucleotide component of the recombinant DNA construct or vector. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the information contained in a given DNA sequence. The process includes both transcription and translation.

A host cell has been "transformed" by exogenous or heterologous DNA when the DNA has been introduced into the cell. Transformation may or may not result in integration (covalent incorporation) into the chromosomal DNA of the cell. For example, in eukaryotic cells such as yeast and mammalian cells, the transfected DNA may be maintained on an episomal element such as a plasmid.

As used herein, the terms "stably transformed", "stably transfected" and "transgenic" refer to cells that have a non-native (heterologous) nucleic acid sequence integrated into the genome. Stable transformation is demonstrated by the establishment of cell lines or clones comprised of a population of daughter cells containing the transfecting DNA.

In some cases "transformation" is not stable, *i.e.*, it is transient. In the case of transient transformation, the exogenous or heterologous DNA is expressed, however, the introduced sequence is not integrated into the genome.

As used herein, the term "co-transformed" refers to a process by which two or more recombinant DNA constructs or vectors are introduced into the same cell. "Co-transformed" may also refer to a cell into which two or more recombinant DNA constructs or vectors have been introduced.

As used herein, the term "adjacent" refers to two sites on a given DNA sequence which in general are separated by less than about 20 nucleotides.

As used herein, the term "flanking compound-binding sequence" means a sequence of from about 8 to 20 nucleotides which is introduced in the vicinity of the DNA response element for a transcriptional regulatory protein. For example, a sequence of from about 8 to 20 nucleotides may be introduced, 3' and 5', respectively, of the transcriptional regulatory protein DNA response element.

As used herein, the term "sequence preferential binding" refers to the binding of a molecule to DNA in a manner which indicates a preference for binding to a certain DNA sequence relative to others.

As used herein, the term "sequence specific binding" refers to the binding of a molecule to DNA in a manner which indicates a strong binding preference for a particular DNA sequence.

As used herein, the term "sequence-dependent binding" refers to the binding of molecules to DNA in a manner that is dependent upon the target nucleotide sequence. Such

binding may be "sequence-preferential" or "sequence-specific."

As used herein, the term "inhibit binding" relative to the effect of a given concentration of a particular compound on the binding of a transcriptional regulatory protein to its DNA response element refers to a decrease in the amount of binding of the transcriptional regulatory protein to its DNA response element relative to the amount of binding in the absence of the same concentration of the particular compound, and includes both a decrease in binding as well as a complete inhibition of binding.

As used herein, the term "regulate a molecular switch" refers to the ability of a DNA binding compound to bind to a nucleic acid sequence in the vicinity of the DNA response element for a transcriptional regulatory protein, thereby modifying the expression of a gene operably linked to the DNA response element.

As used herein, the terms "compound", "molecule", "ligand" and "inducer" are used interchangeably and refer to molecules or ligands characterized by sequence-preferential or sequence-specific binding to DNA at a sequence which is adjacent (*i.e.*, flanking), overlapping, or the same as, the DNA binding site for a transcriptional regulatory protein.

As used herein, the term "dimer" refers to a compound that has two subunits, which are linked to one another and each of which may or may not have the same chemical structure. "Dimers" are a preferred embodiment for compounds used in the methods and compositions of the invention.

As used herein, the terms "modulate" and "modify" are used interchangeably and refer to a change in biological activity. Modulation may relate to an increase or a decrease in biological activity, binding characteristics, or any other biological, functional, or immunological property of the molecule.

The systems of the present invention described herein as systems for "modifying the level of expression of an exogenous gene by a DNA-binding compound", or "regulatable expression systems", are also referred to as "molecular switch systems".

As used herein, the terms "native", "natural" and "wild-type" relative to a particular nucleic acid sequence, trait or phenotype refers to the form in which that nucleic acid sequence, trait or phenotype is found in nature.

As used herein, the term "transgenic plants" refers to plants that have incorporated exogenous nucleic acid sequences, *i.e.*, nucleic acid sequences which are not present in the native ("untransformed") plant or plant cell.

As used herein, the term "T DNA sequence" refers to a sequence derived from the T₁ plasmid of *Agrobacterium tumefaciens* containing the nucleic acid sequence, which is transferred to a plant cell host during infection by *Agrobacterium*.

As used herein, the term "border sequence" refers to the nucleic acid sequence, which corresponds to the left and right edges ("borders") of a T-DNA sequence.

As used herein, a "plant cell" refers to any cell derived from a plant, including undifferentiated tissue (*e.g.*, callus) as well as plant seeds, pollen, propagules and embryos.

As used herein, the term "modified" regarding a plant trait, refers to a change in the phenotype of a transgenic plant relative to a non-transgenic plant, as it is found in nature.

As used herein, the term "*in vitro*" relative to the molecular switch system described herein, refers to cell-based assays carried out *in vitro*, including, but not limited to, binding

and displacement assays and expression assays using reporter genes.

As used herein, the term "*in vivo*" refers to the *in vivo* expression of a transgene using a regulatable molecular switch, as described herein.

5 II. Regulatable Gene Expression/Molecular Switch Systems

A. General Considerations

An effective regulatable gene expression system for use in the methods and compositions of the invention has the following properties: (1) the ability to increase or decrease the expression of a gene of interest, (2) the ability to control the level of expression,
10 and (3) the ability to reduce the potential toxicity of the compound used to induce expression.

B. Expression Systems Induced By Binding To Transcriptional Regulatory Proteins

Many DNA binding transcription factors are comprised of separable DNA binding and transcriptional activation domains. By interchanging DNA-binding and transcriptional
15 activation domains from bacterial, yeast, mammalian, and viral proteins, chimeric regulatory proteins may be developed which have unique specificity and can be regulated in various host cell systems.

Several groups have successfully engineered chimeric regulatory proteins, which are generally composed of a non-mammalian DNA-binding domain and a regulatory domain of
20 either mammalian or non-mammalian origin. A chimeric transcriptional activator with a non-mammalian DNA-binding domain allows activation of a non-mammalian response element in a mammalian system. Depending upon the level of activation required, strong viral or cellular activation domains are used.

Synthetic inducible systems utilizing both prokaryotic and eukaryotic non-mammalian
25 DNA-binding domains have been described in the literature. The present invention makes use of various components of the synthetic inducible systems and chimeric regulatory proteins, as summarized below.

Prokaryotic inducible systems generally make use of prokaryotic repressor/operator systems such as the tet (tetR) or lac (lacI) repressor proteins. The repressor proteins contain
30 domains that bind operator sequences specifically and domains that bind specific exogenous inducers (*e.g.* tetracycline for tetR and IPTG for lacI), and bind their operators in the absence of exogenous inducers that block transcription. In the presence of an exogenous inducer, the repressor binds to the inducer, changing its conformation, resulting in release of the repressor from the operator, and activation of transcription. New synthetic regulatable
35 systems have been developed by fusing the DNA binding and inducer binding domains of these bacterial regulatory proteins to viral transactivation domains (Baim *et al.*, 1991; Gossen and Bujard, 1992).

The purine repressor protein, PurR, is a member of the lac repressor, LacI, family of DNA-binding proteins and binding to the operator of the pur regulon results
40 in negative coregulation of expression. The exemplary native transcriptional regulators of PurR: purF, purFMUT, IHF, and Lef-1 provide potential binding sites for the purR protein, making them targets for regulation of the repressor using DNA-binding compounds.

Further exemplary systems include a synthetic expression system containing a modified CMV promoter with tandem repeats of tetO elements and a fusion protein consisting of a TetR DNA binding domain and a VP16 transactivator. Upon binding of tetracycline or doxycycline to the TetR protein, the chimeric TetR/VP16 protein is released from tetO elements and gene expression is down regulated (tet OFF system). Inducer mediated up-regulation of transcription has been achieved by mutating the TetR such that the mutant TetR (TetR*) binds to tetO elements in the presence of inducers such as tetracycline or doxycycline and up-regulates transcription of the transgene (tet ON system). (Gossen, *et al.*, 1995). The TetR systems lack appropriate pharmacokinetics for rapid temporal regulation in that to reach the maximal activation in the tet ON system, the inducer needs to be cleared from the cells. Following removal, the resumption of full promoter activity takes 48 hours for tetracycline and 216 hours for doxycycline for (A-Mohammadi, *et al.*, 1997).

Also described in the literature are similar synthetic expression systems which are responsive to hormones such as estradiol or RU486. (See, *e.g.*, Wang, *et al.*, 1994; Delort and Capecchi, 1996.) However, the inducers used in these systems, estradiol and RU486, are toxic or abortive.

A further type of regulatable expression system includes a DNA binding unit (ZFHD1/FKB12), and transcriptional activation unit (NF- κ B p65/FRAP, Rivera, *et al.*, 1996), expressed as separate polypeptides which come together in the presence of an exogenous inducer (rapamycin), to function as a response element specific transcriptional activator. Although the synthetic components of the chimeric transactivator are of human origin, and accordingly may be less immunogenic in humans, the inducer, rapamycin, is an immunosuppressive agent.

Non-mammalian eukaryotic elements which have also been utilized to generate chimeric regulators include the yeast *Saccharomyces cerevisiae* Gal4 DNA binding domain (Brasemann *et al.*, 1993; Wang *et al.*, 1994) or Leu3 (Guo and Kohlhaw, 1996) has been fused with various regulatory domains. For example, a fusion protein consisting of the Gal4 DNA binding domain, the estrogen receptor or the mutated progesterone receptor ligand binding domain and the VP16 transactivation domain may be regulated by exogenous estradiol or RU486, respectively (Whelan and Miller, 1996; Wang *et al.*, 1994). Several variations of this basic system have been described (Whelan and Miller, 1996).

The insect hormone ecdysone inducible expression system (No *et al.*, 1996), is based on a chimeric ecdysone receptor/VP16 fusion protein which dimerizes with the retinoid X receptor in the presence of ecdysone or its synthetic analogue, muristerone. The dimerized receptor binds the ecdysone response element and acts as transcriptional activator.

A further type of regulatable expression system includes a DNA binding domain and transcriptional activation domain expressed as separate polypeptides, and which come together in the presence of an exogenous inducer to function as a response element specific transcriptional activator. An exemplary construct includes, as a DNA binding domain, ZFHD1 (a synthetic fusion protein that contains zinc fingers 1 and 2 from Zif268, a short polypeptide linker, and the homodomain of Oct-1; Pomerantz *et al.*, 1995), fused to the human protein FKB12, and the p65 activation domain of the human transcription factor NF- κ B fused to another human protein FRAP (Rivera *et al.*, 1996). Although the synthetic

components of the chimeric transactivator are derived from human origin, and accordingly may be less immunogenic in humans, the inducer, rapamycin, is an immunosuppressive agent.

None of the aforementioned regulatable expression systems exhibit all the features of an effective regulatable gene expression system. The TetR system lacks pharmacokinetics necessary for a tightly controlled system. In addition, systems such as TetR are not applicable to agricultural applications, in that it is not practical for an inducer (*i.e.* tetracycline) to be sprayed on an entire field of plants.

The hormone (estradiol or RU486) and rapamycin-inducible systems suffer from toxicity problems with the specific compounds used to induce expression. Further, in the ecdysone system and the rapamycin inducible system, two chimeric proteins need to be expressed in order to make the chimeric transcription factor.

C. Expression Systems Induced By Binding To DNA

All of the aforementioned regulatable expression systems utilize compounds (inducers) that act on protein transcriptional factors. The binding of a compound or inducer to a transcriptional regulatory protein appears to change the conformation of the protein, which leads to the changes in either the DNA binding property or the dimerization property of the factors, resulting in changes in the regulatory properties of the chimeric regulator. The fact that prior art protein-inducible systems require a compound which is specific to the inducer domain of the transcriptional regulatory protein significantly limits the choice of compounds capable of functioning as inducers in a given system. Any DNA binding compound that modulates the binding of the transcriptional regulatory protein can be utilized as an inducer in the molecular switch systems of the present invention. In both switch-on and switch-off systems, described herein, the incorporation of compound-binding sequences in the vicinity of the DNA response element for a transcriptional regulatory protein permits a wide selection of compounds effective to regulate the expression of genes operably linked to such a response element. However, it will be understood that in some cases the compound-binding sequence and the DNA response element for the transcriptional regulatory compound have the same sequence.

The present invention is directed to a molecular switch system utilizing a transcriptional regulatory protein and an exogenously supplied compound, which targets nucleic acid, not protein. It has been well established through the Merlin™ technology that DNA binding compounds, when bound to double stranded DNA at sites in the vicinity of regulatory protein binding sequences, can displace the bound protein. See, *e.g.*, U.S. Pat. Nos. 5,306,619, 5,693,463, 5,716,780, 5,726,014, 5,744,131, 5,738,990, 5,578,444, and 5,869,241, expressly incorporated reference herein.

III. Methods And Compositions Of The Invention

In the molecular switch methods and compositions of the invention, when a transcriptional regulatory protein DNA binding site is in the vicinity of (the same as, overlapping or adjacent to), a compound-binding site, the binding of the transcriptional regulatory protein may be controlled by an exogenous DNA binding compound.

A. Embodiments Of The Molecular Switch System

A number of embodiments of the molecular switch systems of the invention may be used to regulate gene expression. In its basic form, the molecular switch system includes a nucleic acid construct which has a compound-binding site in the vicinity of (the same as, overlapping or adjacent to), the DNA response site for a transcriptional regulatory protein, a DNA binding compound and a transcriptional regulatory factor (Figure 1). Transcriptional regulatory factors or proteins for use in the molecular switch systems of the invention may be one or more of (1) endogenous, (2) exogenously supplied, (3) native, (4) synthetic (engineered), (5) chimeric, (6) effective in specific tissues or cell types, and (7) effective in a tissue or cell type independent manner.

The components of the molecular switch system of the invention may be provided to a cell by way of one or two vectors.

In one exemplary one vector embodiment of the invention, the transcriptional regulatory protein may be a native endogenous protein. In such cases, the vector comprises a synthetic DNA response element for the transcriptional regulatory protein which has a compound-binding sequence in the vicinity of the DNA response sequence and a transgene under the control of a first promoter.

In another one vector embodiment, an engineered transcriptional regulatory protein is exogenously provided to a cell in the same vector construct as a synthetic DNA response element and associated compound-binding sequence. In this aspect, the vector comprises a synthetic DNA response element for the transcriptional regulatory protein which has a compound-binding sequence in the vicinity of the DNA response sequence and a transgene under the control of a first promoter and the coding sequence for an engineered transcriptional regulatory protein under the control of a second promoter.

In still other cases, a single vector is effective to express both a transcriptional regulatory protein and a transgene under the control of a single compound-inducible promoter, utilizing IRES.

In one exemplary two vector embodiment of the invention, the first vector comprises the synthetic DNA response element for a transcriptional regulatory protein which has a compound-binding sequence in the vicinity of the DNA response element and a transgene under the control of a first promoter and the second vector comprises the coding sequence for an engineered transcriptional regulatory protein operably linked to a second promoter.

In some cases, the expression of the engineered transcriptional regulatory protein may also be regulated by a compound. In such cases, the construct has a compound-binding sequence in the vicinity of the DNA response element for a transcriptional regulatory protein and a second promoter operably linked to the coding sequence for the engineered transcriptional regulatory protein. In such cases, the first and second vectors may or may not have the same compound-binding sequence and DNA response element.

In such two vector embodiments, when the transcriptional regulatory protein is engineered, it may be an exogenously supplied native protein, it may be synthetic or chimeric, and may be effective in specific tissues or cell types, or may be effective in a tissue or cell type independent manner.

In both the one and two vector embodiments of the molecular switch system, the invention includes a compound or inducer, which when bound to a compound-binding sequence is effective to modify expression of a gene under control of the promoter.

In a chimeric activator DNA binding compound-mediated molecular switch system, the binding of a compound directly to, adjacent, or overlapping the DNA binding site for a transcriptional regulatory protein displaces the bound transcriptional regulatory protein from the DNA response element of a promoter. In such cases, the displacement of the transcriptional regulatory protein leads to down-regulation of transcription of an operably linked transgene (switch-off system).

A similar system which is switched-on by binding of a compound includes a chimeric transcriptional regulatory protein with a repressor domain instead of a transactivator domain.

Incorporation of a strong activator or repressor domain into an engineered transcriptional regulatory protein confers a wide range of activity to the regulatory protein in a regulatable gene expression construct. By incorporating promoters that function in a variety of cell types into vector constructs which have an appropriate DNA response element, expression can be achieved in the particular cell types.

In the methods of the invention, cell lines which produce a given transcriptional regulatory protein may be generated and transformed with vector constructs having a variety of compound-binding sequences. A repertoire of different regulatable expression systems may then be generated using the same basic transcriptional regulatory protein construct and DNA response element, by modifying the number of copies (repeats) of the DNA response element, and by the use of different compound-binding sequences.

In one embodiment, the system involves a natural transcriptional regulatory factor (protein) that is either tissue-specific, disease-specific or heterologous and unique to the host. Such natural or native factors may be provided exogenously or may be endogenous to a particular tissue, cell or host. In either case, such a natural DNA-binding regulatory factor will bind to a synthetic DNA response element which has been introduced into cells and has a compound-binding sequence which is the same as, overlapping, or adjacent to the DNA response element. A synthetic DNA response element for one or more natural factors may be provided to a cell.

As set forth above, in another embodiment, the system incorporates engineered regulatory proteins (activators or repressors), which are provided to cells together with a corresponding synthetic DNA response element and associated compound-binding sequence. It will be understood that the DNA sequence encoding an engineered transcriptional regulatory protein is exogenously supplied, it may be provided in the same or in a different vector construct as the synthetic DNA response element and associated compound-binding sequence. In addition, the expression of an engineered regulatory protein may be under the control of a constitutive promoter or a compound inducible promoter. When the expression of an engineered regulatory protein is under the control of a compound inducible promoter, expression may be induced by a compound which is the same as, or differs from, the compound which binds a sequence in the vicinity of the DNA response element for the regulatory protein.

Regulatable gene expression systems may be designed wherein the compound-binding

sequence and the regulatory protein binding site are the same. In such cases, a native endogenous regulatory protein is used or alternatively, an exogenous, synthetic regulatory protein may be "designed" which has a DNA-binding domain which specifically binds the compound-binding sequence/transcriptional regulatory protein binding site. (See, *e.g.*,
5 Greisman and Pabo, 1997, which describes the selection of novel zinc three-finger proteins which bind to a specific 9 to 10 bp sequence.)

It will be understood that in some cases the DNA response element for a given transcriptional regulatory protein will include a site that also functions as the preferential binding sequence for a DNA-binding compound, *i.e.*, a small molecule. In such cases, the
10 DNA response element may be incorporated into the regulatable expression system of the invention in a single copy or constructs may be engineered including one or more tandem repeats of the sequence.

In other cases, the promoter sequence in the vicinity of the DNA response element will be modified to include one or more preferred binding sequences for a DNA-binding
15 compound resulting in a regulatable promoter construct.

In one preferred embodiment, a single vector molecular switch system is employed wherein the vector contains a transgene under the control of a promoter operably linked to the DNA response element for a native transcriptional regulatory protein which has a compound binding site in the vicinity of the DNA response element. A luciferase reporter
20 gene may be used to evaluate regulatable gene expression *in vitro* in cell culture. However, any reporter gene known to those of skill in the art may also be used (as further described below).

Once the ability of a compound to displace a transcriptional regulatory protein from its DNA response element has been demonstrated in a cell-based assay using a reporter
25 construct, the genetic construct may be readily modified to include a gene of interest, such as a therapeutic gene, recombinant protein-encoding gene or drug resistance gene, in place of the reporter gene. Such modifications may be made using techniques routinely used by those of skill in the art.

In cases where the molecular switch system takes advantage of natural regulatory proteins or factors, *i.e.*, those having tissue specificity or disease specificity, the genetic
30 construct may deliver a therapeutic gene under control of an inducible promoter with multiple natural factor response elements flanked by compound-binding sequences without a need for an exogenous regulatory protein.

Alternatively, a natural promoter may be modified to include one or more compound
35 binding sequences near the natural factor binding sites in the promoter, *e.g.*, NF-KB and TFIID sites in a modified CMV promoter.

When the molecular switch system employs an exogenous transcriptional regulatory protein, the regulatory protein is supplied along with therapeutic gene, either in a single genetic construct or in separate genetic constructs.

40 An exogenous regulatory protein gene and a therapeutic gene may be placed under the control of the same compound-inducible promoter, and delivered by a single vector, *e.g.*, by placing an internal ribosomal entry site in front of the synthetic activator gene. In such cases, the compound not only displaces the exogenous regulatory protein, *e.g.*, activator,

from the promoter, down-regulating the expression of the therapeutic gene, it also reduces the expression of the activator protein, providing a system with tighter regulation.

In summary, the molecular switch system provides single vector embodiments comprising one or more promoters and two vector embodiments, each comprising a promoter which may be the same or different.

Once the one or more binding sites for such an essential transcriptional regulatory protein are determined, compound binding sequence(s), *e.g.* for a small molecule, are engineered into the promoter near the transcriptional regulatory protein DNA response element(s) and thereby used to regulate the binding of the transcriptional regulatory protein to the promoter, resulting in regulation of promoter activity.

For example, an engineered promoter that is regulated by a DNA binding molecule can be created. In one example, a sequence comprising from about 1 to 12 or more tandem repeats of the NF-kB site with a corresponding number of compound binding sequences in the vicinity of the NF-kB site is added to a CMV minimal promoter sequence (Example 2).

Alternatively, the DNA response element for more than one type of transcriptional regulatory factor may be incorporated into a single promoter, particularly when the selected transcriptional regulatory factors work cooperatively.

In a further embodiment, a natural tissue-specific promoter is modified to include one or more introduced compound binding sequences near one or more natural transcriptional regulatory factor binding sites which are essential for transcriptional regulation of the natural tissue-specific promoter.

Temporal and spatial regulation of gene expression can be achieved by combining the tissue specificity of such a promoter with regulation of the interaction between the tissue-specific promoter and one or more essential transcriptional regulatory proteins, by the exposure of the promoter to a DNA binding compound which exhibits sequence-preferential binding to the introduced compound binding sequence(s).

A synthetic promoter may be made by introducing one or more tissue-specific transcription factor binding sites and one or more compound binding sequences into the sequence of a tissue-specific regulatable promoter such that the promoter may be regulated by a compound which preferentially binds the compound binding sequence(s), *e.g.*, a small molecule. Such a small molecule may target an essential transcription factor or tissue specific transcription factor if it is essential to the activity of the promoter.

For example, a CMV/HBV enhancer II hybrid promoter (Sandig, *et al.*, 1996; Loser, *et al.*, 1996), which displays liver specificity, may be modified to have compound-binding sequences in the vicinity of (*i.e.*, adjacent to, or overlapping), essential transcription factor binding sites, such as C/EBP, HNF-1, HNF-3 and SP-1 and/or TATA box.

In another example, tandem repeats of the myocyte-specific enhancer factor 2 (MEF2, SEQ ID NO:22) binding sequence may be fused to the sequence of a CMV minimal promoter to give muscle specificity. MEF2 sites, which are present in many muscle genes (Brand NJ, 1997), may be preferentially targeted by a small molecule such as 21x, given that the MEF2 sequence is "AT-rich".

B. Components of the Molecular Switch System

In all of the embodiments described above, the DNA response site for a transcriptional regulatory protein may contain from 1 to 12 copies of a given response sequence, with multiple copies facilitating amplification of the response. In addition, in each embodiment, natural factor and synthetic factor DNA response sites may be the same as, overlapping, or adjacent to compound-binding sequences. Accordingly, nucleic acid constructs for use in the molecular switch system of the invention may have a compound-binding sequence on one or both sides of each transcriptional regulatory protein DNA response element. Such compound-binding sequences are introduced into the DNA response element of a regulatable expression construct, allowing induction by a DNA binding compound and modulation of the activity of a promoter operably linked thereto.

It will be understood that the various components of the molecular switch systems of the invention are interchangeable. For example, a given regulatory domain may be combined with any of a number of DNA binding domains in a synthetic transcriptional regulatory protein. Similarly, any of a number of DNA response elements which bind a given transcriptional regulatory protein may be used. Many such regulatory domains, DNA binding domains and corresponding DNA response elements are known to those of skill in the art, and are summarized below. DNA binding proteins which affect transcription, but lack a regulatory domain also find utility in the methods of the invention. In general, multiple copies of a transcriptional regulatory protein may bind to its corresponding DNA response element.

Synthetic or engineered transcriptional regulatory proteins for use in the methods and compositions of the invention include a mammalian or a non-mammalian DNA binding domain and a regulatory domain of choice. Synthetic regulatory proteins can be designed by consideration of the DNA response elements for the DNA binding domain and the activity of the transcriptional regulatory protein. Activators or repressors can be used for switch-off or switch-on system, respectively.

In some cases, one or more natural transcriptional regulatory proteins may be employed in the methods and compositions of the invention to facilitate regulated gene expression, such as, homologous, heterologous, host-, tissue- or disease-specific expression. In such cases, a compound-binding sequence is inserted into a nucleic acid construct and is the same as, overlapping, or adjacent to the DNA response element(s) for the one or more natural transcriptional regulatory proteins. For example, a nucleic acid construct which has introduced compound-binding sequences in the vicinity of the TFIID and NF- κ B DNA response elements in a CMV promoter.

C. Transcriptional Regulatory Proteins

In the molecular switch systems of the invention, the choice of DNA binding domain in a given transcriptional regulatory protein will determine the appropriate response element. Different DNA response elements can be utilized together with a corresponding DNA binding transcriptional regulatory protein, and need not have sequence homology to the associated compound binding sequence. The sequences of a number of DNA binding transcriptional regulatory proteins and corresponding response elements are known in the art

and examples are provided in Table 1.

Table 1. Non-mammalian DNA binding proteins and their response elements

<u>DNA BINDING PROTEIN</u>	<u>RESPONSE ELEMENT</u>
TetR (prokaryotic)	tetO (SEQ ID NO:5)
LacR (prokaryotic)	lacO (SEQ ID NO:6)
GAL4 (yeast)	GAL4 (SEQ ID NO:2)
Ecdysone receptor	Ecdysone (SEQ ID NO:7)
ZFHD1 (mammalian)	ZFHD1 (SEQ ID NO:3)
UL9 (viral)	UL9 (SEQ ID NO:1)

Activator and repressor protein domains which may be incorporated into engineered transcriptional regulatory proteins for use in the methods and compositions of the invention may be of mammalian, plant, *Drosophila*, yeast, bacterial, or viral origin, if, when linked to a DNA binding domain, the domain functions as an activator or repressor, respectively when an appropriate DNA response element is introduced into the host cells of the regulatable expression system.

In one embodiment of the regulatable expression system of the present invention, an engineered transcriptional regulatory protein is provided which includes a strong sequence specific activator, UL9-VP16, which has the C-terminal DNA binding domain of UL9 fused to the N-terminus of the activation domain of VP16 utilizing pGEX-UL9 (Genelabs) and pACT (Promega), expressed under the control of a CMV immediate early enhancer/promoter.

In another embodiment, an engineered transcriptional regulatory protein is provided which includes the UL9 C-terminal DNA binding domain fused to the N-terminus of activation domain of NF- κ B p65, prepared by replacing the VP16 domain in the UL9-VP16 construct, with the activation domain of NF- κ B p65 (SEQ ID NO:4).

In a further preferred embodiments, the UL9 C-terminal DNA binding domain is fused to the N-terminus of the repressor domain of kruppel protein (KRAB which is present in about one third of the vertebrate Kruppel-type zinc finger factors (Margolin JF, *et al.*, 1994), or Mad protein (Ayer *et al.*, 1996).

D. Activators

Polypeptides which can function to activate transcription in eukaryotic cells are well known in the art. In particular, transcriptional activation domains of many DNA binding proteins have been described and have been shown to retain their activation function when the domain is transferred to a heterologous protein. Activator domains which may be incorporated into chimeric transcriptional regulatory proteins for use in the methods and compositions of the invention, include but are not limited to VP16, NF-KB, TFE3, ITF1, Oct-1, Sp1, Oct-2, NFY-A, ITF2, c-myc, and CTF (Seipel, *et al.*, 1992).

An exemplary polypeptide for use in a transcriptional regulatory protein of the

invention is the herpes simplex virus virion protein 16, referred to herein as VP16, the amino acid sequence of which is disclosed in Triezenberg, *et al.*, 1988. In one embodiment, amino acids from about 413-489 of the C-terminus of VP16 (SEQ ID NO:8) are used as the transactivator domain (Sadowski, *et al.* 1988). In another embodiment, a tetramer of amino acids 437-447 of VP16 (SEQ ID NO:9) is used as the transactivator domain (Beerli, *et al.*, 1998).

E. Repressors

Native repressors such as LacR or TetR may also be utilized in the molecular switch system of the invention. Such repressors are provided exogenously as one component of a transcriptional regulatory protein, together with a regulatable promoter which has been modified to include one or more compound-binding sequences in the vicinity of (the same as, overlapping, or adjacent to), the DNA response element for a given transcriptional regulatory protein.

Exemplary repressor proteins and their corresponding DNA binding domains for use in the methods and compositions of the invention are summarized in Table 2. The repressor domains include Kruppel (KRAB; Margolin *et al.*, 1994), kox-1 (Deuschle *et al.*, 1995), even-skipped (Licht *et al.*, 1994), LacR, engrailed (Li *et al.*, 1997), hairy (HES; Fisher *et al.*, 1996), Groucho (TLE; Fisher *et al.*, 1996), RING1 (Satjin *et al.*, 1997), SSB16 and SSB24 (Saha *et al.*, 1993), Tup1 (Tzamarlas, Struhl, 1994), Nab1 (Swirnoff *et al.*, 1998), AREB (Ikeda *et al.*, 1998), E4BP4 (Cowell & Hurst, 1996), HoxA7 (Schnabell *et al.*, 1996), EBNA3 (Bourillot *et al.*, 1998), and v-erbA (Busch *et al.*, 1997).

Further exemplary repressors for use in the methods and compositions of the invention include the basic helix-loop-helix (bHLH) proteins (a family of transcription factors, which act as dimers, with their selective dimerization affecting cell proliferation, differentiation or apoptosis), such as Mxi (which is involved in repressing transcription of c-myc-responsive genes, Fisher F *et al.*, 1993); Mnt (Soucek L, *et al.*, 1998), Rox (Takahashi T *et al.*, 1998), and TFEC (Rehli M *et al.*, 1999); the homeoproteins (transcription factors known to exist in all eukaryotes where they perform important functions during development) such as Msx-1 (Stelnicki EJ *et al.*, 1997), Evx1 (Briata P, *et al.*, 1997) and HoxC6 (or Hox-3.3-encoded homeoprotein, Jones FS, 1993); Zn finger proteins such as CTCF (Delgado MD *et al.*, 1999), AREB, Ikeda *et al.*, 1998, REST (zinc finger protein RE-1-silencing transcription factor, Thiel G *et al.*, 1998), EGR-4 (Zipfel PF *et al.*, 1997) and KOX1 (which contains a KRAB domain, Moosmann P *et al.*, 1997); in addition to CDP/cut (human homeodomain CCAAT displacement protein/cut homolog, Li S *et al.*, 1999; Mailly F *et al.*, 1996); ATF-3 (Wolfgang CD *et al.*, 1997); MBP (Ghosh AK *et al.*, 1999); BP1 (Berg PE *et al.*, 1991); ERF (Day RN *et al.*, 1998); Dr1 (White RJ *et al.*, 1994), MeCP2 (methyl Cp-G-binding protein; Nan X *et al.*, 1998); ZFM1 (human zinc finger motif 1, Zhang D *et al.*, 1998), BERF-1 (Antona V *et al.*, 1998); PRDI-BF1/Blimp-1 protein (Ren B *et al.*, 1999), IFI 16 (interferon-inducible transcriptional repressor, Johnstone RW *et al.* 1998), ICER (inducible cAMP early repressor, Bodor J *et al.*, 1998), COUP TF (Chicken ovalbumin upstream promoter-transcription factor, Bailey PJ *et al.*, 1997); DAX-1 (Zazopoulos E *et al.*, 1997), ATF3 [in the activating transcription factor/cAMP responsive element binding protein

(ATF/CREB) family of transcription factors, Wolfgang CD *et al.*, 1997], and polyhomeotic protein (Ph, Satijn DP *et al.*, 1997).

Table 2. Repressors with tethering DNA binding domain

<u>Repressor</u>	<u>Origin</u>	<u>DNA binding domain</u>	<u>Reference</u>
kruppel	Drosophila	Gal4	Margolin <i>et al.</i> , 1994
kox-1	Human	TetR	Deuschle <i>et al.</i> , 1995
even-skipped	Drosophila	LacR	Licht <i>et al.</i> , 1994
engrailed	Drosophila	Qin	Li <i>et al.</i> , 1997
hairy (hes)	Drosophila (human)	Gal4	Fisher <i>et al.</i> , 1996
Groucho(TLE)	Drosophila (human)	Gal4	Fisher <i>et al.</i> , 1996
RING1	Drosophila	LexA Gal4	Satjin <i>et al.</i> , 1997
SSB16 SSB24	<i>E.coli</i>	Gal4	Saha <i>et al.</i> , 1993
Tup1	Yeast	LexA	Tzamaras Struhl, 1994
Nab1	Human	Gal4	Swirnoff <i>et al.</i> , 1998
AREB	Human	Gal4	Ikeda <i>et al.</i> , 1998
E4BP4	Human	Gal4	Cowell & Hurst, 1996
HoxA7	Mouse	Gal4	Schnabell <i>et al.</i> , 1996
EBNA3	EBV	Gal4	Bourillot <i>et al.</i> , 1998
v-erbA	virus	Gal4	Busch <i>et al.</i> , 1997
Mad	Mammalian	Gal4	Ayer <i>et al.</i> , 1996

F. DNA Response Elements

In the molecular switch system described herein, the DNA response element which binds the transcriptional regulatory protein may be of mammalian or non-mammalian origin and is generally present in multiple (about 1 to 12) copies, as tandem repeats.

For example, the transcriptional regulatory protein DNA response sequence may be a UL9 sequence, an NF- κ B sequence or a LacR sequence which is present as 1 to 12 tandem repeats. (See Examples 1, 2 and 3.)

Preferred DNA response sequences for use in the methods and compositions of the invention are UL9, NF- κ B, GAL4, ZFHD1, LacR, TetR, LexA, the UP element of *rrnB* P1, and the ecdysone receptor binding sequence. However, it will be understood that the DNA response sequence for any known DNA-binding protein may be incorporated into the regulatable gene expression systems of the invention. Such a DNA-binding protein, may or may not contain an activator or repressor domain.

G. Promoters

The choice of promoter can significantly affect both temporal and spatial aspects of gene expression. Strong promoters with enhancers may result in a high level of expression. However, when a low level of basal activity is desired, a weak promoter may be a better choice. Expression of transgenes of interest may also be controlled at the level of transcription, by the use of cell type specific promoters or promoter elements in gene transfer vectors. Exemplary cell type specific promoters/elements and their target cell/tissue specificity are provided in Table 3. (See also, Walther and Stein, 1996; Miller and Whelan, 1997).

Table 3. Promoters with tissue specificity

Gene Promoter	Target cell/tissue
	<u>Hematopoietic cells</u>
CD11a	Leukocytes
CD 11b	Leukocytes
CD18	Leukocytes
β -Globin promoter/LCR	Erythroid cells
Immunoglobulin promoters	B-lymphoma
Human parvovirus B19	Erythroid cells
Scavenger receptor A	Macrophages, foam cells
Glycoprotein IIb	Megakaryocytes, platelets
γ c chain	Mature myeloid cells
	<u>Brain</u>
	<u>Liver, intestine and kidney</u>
PEPCK	Hepatocytes
Albumin	Hepatocytes
hAAT	Hepatocytes
HBV	Hepatocytes
Fatty acid synthetase	Liver, adipose tissue
Factor VII	Liver
Carbamoyl phosphate Synthetase I	Portal vein hepatocytes
Na-K-Cl transporter	Small intestine
	Kidney
	<u>Mammary gland</u>
MMTV-LTR	Mammary carcinoma
WAP	Mammary carcinoma
β -casein	Mammary carcinoma
	<u>Epithelium and endothelium</u>
SPC	Broncheolar and alveolar epithelium
SP-A	Broncheolar and alveolar epithelium
SP-B	Broncheolar and alveolar epithelium
E-cadherin	Epithelium
Flt-1	Endothelial cell
Preproendothelin	Endothelium, epithelium, muscle
	<u>Keratinocytes and others</u>
Cytokeratins	Keratinocytes
Transglutaminase 3	Keratinocytes
Bullous pemphigoid antigen	Basal keratinocytes

Keratin 6	Proliferating epidermis
Collagen α 1	Hepatic stellate cells skin/tendon fibroblast
Type X collagen	Hypertrophic chondrocytes
	<u>Muscle</u>
MCK	Undifferentiated myogenic cells
VLC1	Myoblasts
GLUT4	Skeletal muscle
Slow/fast troponins	Slow/fast twitching myofibers
α -actin	Smooth muscle
myosin heavy chain	Smooth muscle
	<u>Virus infected cells</u>
HIV-LTR	HIV infected Lymphocytes
Tat/Rev-responsive elements	HIV infected CD4 + T-cells
Tat-inducible element	HIV infected CD4 + T-cells
EBNA-1	EBV infected cells
	<u>Cancer</u>
PSA	Prostate
Aromatase	Cancer
CEA	Colon and lung carcinomas
AFP	Hepatocellular carcinomas
SLPI	Carcinomas
Tyrosinase	Melanomas
Varicella Zoster virus	Melanocytes
c-erbB2	Breast, pancreatic, gastric carcinomas
	Lung cancer
Myc-Max responsive element	Ras-transformed cells
Murine parvovirus MVMp	
	<u>Pathological milieu</u>
Egr-1	Irradiated tumors
Grp78	Anoxic, acidic tumors
MDR1	Tumors treated with chemotherapy
HSP70	Tumors treated with hyperthermy
VEGF	Hypoxic angiogenesis
Nitric oxide synthase	Hypoxic angiogenesis
Murine CF3	Liver, lung inflammation
Serum amyloid 3	Liver inflammation
Bovine keratin 6	Hyperproliferating epithelial cells

The promoter component of the heterologous nucleic acid constructs for use in the molecular switch systems of the invention may be a minimal or full length promoter sequence. An exemplary engineered or synthetic promoter may comprise a minimal promoter sequence fused to a cis element, such as an endogenous DNA response element for: NF- κ B, myocyte-specific enhancer factor (MEF), or hepatic nuclear factor (HNF); or alternatively a bacterial sequence such as LacO, or a viral sequence such as UL9.

Preferred constitutive promoters for use in the methods and compositions of the invention include any of a number of promoters known to those of skill in the art, examples of which are a minimal CMV promoter, a CMV immediate/early enhancer promoter, an SV40 promoter, the HSV TK promoter, the MuLV LTR promoter and the HIV LTR promoter. Such promoters may be used in the native form in conjunction with natural transcriptional regulatory proteins or may be modified to include the DNA response elements for a natural or synthetic transcriptional regulatory protein.

In molecular switch systems which utilize either synthetic or natural transcriptional regulatory proteins, promoter activity may be amplified by incorporating tandem repeats of the appropriate DNA response element into the regulatable gene expression system.

Promoter activity may be further amplified by the use of an enhancer sequence, *e.g.*, SV40, HIV or CMV enhancer sequences.

H. Compound binding sites

Compound-binding sequences are generally 8-20 bp in length and may be the same as, overlapping, or adjacent to the DNA response element for a transcriptional regulatory protein.

In one embodiment, the sequences are inserted next to either one or both ends of a transcriptional regulatory protein DNA response element.

In another embodiment, the compound binding sequences overlap a transcriptional regulatory protein DNA response element.

In the case of transcriptional regulatory protein response sites which consist of repeated sequence portions, the compound-binding sequence may flank each repeated sequence portion, or may flank the entire transcriptional regulatory protein response site.

In both repressor- and activator-mediated systems, incorporating compound-binding sequences in the vicinity of the DNA response element for a given transcriptional regulatory protein permits a wide selection of inducers.

Typically, binding of a DNA-binding compound to a compound-binding sequence interferes with the binding of a transcriptional regulatory protein to its corresponding DNA response element. However, the binding of some DNA-binding compounds to such DNA response elements may have the opposite effect, causing increased binding of the transcriptional regulator, *i.e.*, activator, under conditions effective to result in expression of a transgene operably linked thereto.

In addition, each embodiment set forth above further includes one or more compound binding sequences in the vicinity of the DNA response element, as exemplified by an 8 to 20 or more bp "AT-rich" sequence which is the preferred binding preferred binding sequence for the netropsin dimer, designated 21x.

I. Transgenes

When evaluating the affect of the molecular switch system on transcription in cell based *in vitro* screening assays, selection of the reporter gene, determines the assay format. For example, luciferase activity can be measured by biochemical reaction with lysates from transfected cells followed by using a luminometer. If the green fluorescence protein is used

as reporter, cells can be directly monitored for their fluorescence without biochemical assay, and transformed cells can be separated easily by FACS, which facilitates faster selection and enrichment of transformed cells compared to conventional methods which involve antibiotic selection.

5 Preferred reporter genes for use in the methods and compositions of the invention include, luciferase, green fluorescent protein (GFP), blue fluorescent protein (BFP), CAT, β -galactosidase, human growth hormone, alkaline phosphatase, etc., under the control of an appropriate promoter.

10 In nucleic acid constructs for use in cell-based reporter assays using the molecular switch system set forth above, the DNA response element for the transcriptional regulatory protein has from 1 to 12 copies of the DNA response element for the transcriptional regulatory protein, together with a promoter and a reporter gene, *e.g.*, luciferase.

15 In one exemplary embodiment, a luciferase reporter construct with a series of tandem repeated UL9 binding sites and flanking compound-binding sequences is made by modification of the pG5luc vector (Promega). In this construct, the firefly luciferase is under the control of a synthetic promoter that is composed of five tandem repeats of the GAL4 binding site followed by the site for the major late minimal promoter of adenovirus. For use in the methods of the present invention, the Gal4 binding sites in the vector are replaced with 1 to 12 copies of the UL9 binding site, flanked by 21x binding sequences.

20 IV. Introduction Of Nucleic Acid Constructs Into Cells

A nucleic acid construct for use in the molecular switch system of the invention is introduced into either eukaryotic or prokaryotic cells. In the case of engineered, synthetic and heterologous native transcriptional regulatory proteins, a vector encoding the protein is introduced into a host cell, wherein the nucleic acid is in a form suitable for expression of the protein in that host cell. For example, a recombinant expression vector of the invention, encoding the protein, is introduced into a host cell.

30 A "host cell" includes any cell or cell line which is not incompatible with the protein to be expressed, the selection system chosen or the fermentation system employed. Host cells for use in the molecular switch systems of the invention include human cells, other non-human mammalian cells, yeast, bacteria, insect cells, plant cells, archaea, fungi, etc.

In addition to cell lines, the invention is applicable to normal cells *in vitro*, *ex vivo* and *in vivo*, such as cells to be modified for gene therapy purposes, embryonic cells modified to create a transgenic or homologous recombinant animal, and plant cells.

35 Methods known in the art for delivery of nucleic acid constructs into mammalian cells include viral methods using adenoviral vectors, retroviral vectors, or adeno-associated viral vectors; non-viral methods using plasmids, liposomes, or other vehicles; and physical or chemical methods using calcium phosphate transfection or gene gun techniques.

40 Similarly, methods known in the art for delivery of a nucleic acid construct into plant cells include bacterial vectors such as the Agrobacterium Ti vector, and viral vectors such as the tomato mosaic virus and potato X virus.

In addition, baculovirus vectors may be used to deliver a nucleic acid construct into insect cells, and bacteria may be transformed with plasmids, and phage such as lambda

phage.

For example, vectors encoding transcriptional regulatory proteins can be introduced into a host cell by standard techniques for transfecting cells. The term "transfecting" or "transfection" is intended to encompass all conventional techniques for introducing a nucleic acid construct into a host cell, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation and microinjection. Suitable methods for transfecting cells can be found *e.g.*, in Sambrook, *et al.*, 1989, expressly incorporated by reference herein.

The number of host cells transformed with a nucleic acid construct of the invention will depend, at least in part, upon the type of recombinant expression vector used and the type of transfection technique used. Nucleic acid can be introduced into a host cell transiently, or more typically, for long term regulation of gene expression, the nucleic acid is stably integrated into the genome of the host cell or remains as a stable episome in the host cell. Plasmid vectors introduced into mammalian cells are typically integrated into host cell DNA at only a low frequency. In order to identify these integrants, a gene that contains a selectable marker (*e.g.*, drug resistance) is introduced into the host cells along with the nucleic acid of interest, and the transfected cells are cultured in medium containing the appropriate drug. Preferred selectable markers include neomycin, zeomycin and hygromycin.

In some cases, two separate plasmids may be used to deliver a transcription factor and a transgene into a cell; one or both of which are under the control of regulatable or constitutive promoters. In such cases, the same compound may be used to regulate the expression of both the transcriptional regulatory protein and the transgene, which may result in feedback regulation.

In an exemplary embodiment of the method of the invention, HeLa, COS, MCF7 or HepG2 cells are transfected with an expression vector encoding a synthetic transcriptional activator protein under conditions effective to generate transformants which express the transcriptional activator. Expression of the activator is monitored by Western blot or Northern.

Once transformants expressing the transcriptional regulatory protein have been generated, they are transfected with vector constructs having different numbers of UL9 DNA binding sites, and co-transfected with a copy control, *e.g.*, a Renilla luciferase plasmid.

In some cases, cells are co-transfected with plasmids containing: (1) nucleic acid sequences for expression of an engineered transcriptional regulatory protein, (2) nucleic acid sequences which have various different numbers of transcriptional regulatory protein DNA binding sites, and (3) nucleic acid sequences which serve as a copy number control at the same time.

The luciferase activity of transformants is measured and constructs selected which have an operable number of UL9 binding sites selected, *i.e.*, constructs which give detectable luciferase activity are selected. Molecular switch constructs for use in the methods and compositions of the invention are generated by adding compound-binding sequences in the vicinity of the DNA response element for the transcriptional regulatory protein to constructs having an operable number of DNA response elements for the transcriptional regulatory

protein.

Transformants that express a transcriptional regulatory protein are transfected with promoter constructs which have a response site and a copy control reporter plasmid, followed by treatment with different amounts of appropriate compounds. The effect of the compound on reporter (*e.g.*, luciferase) activity is then determined. In most cases, the initial assay is done with transiently transfected cells. In such cases, double stable transformants are made later and the activity is verified.

Reporter constructs are used to identify and optimize operable nucleic acid constructs for use in the molecular switch systems of the invention. Once the components of the system have been engineered and tested in the context of reporter constructs, the reporter is generally replaced by a transgene which encodes a protein or polypeptide of interest.

It will be understood that following engineering, optimization and testing, the components of the molecular switch system are then transferred to vectors appropriate to the application, *e.g.* gene therapy vectors or vectors for expression in plant cells.

V. Compounds (Inducers)

Small molecules are desirable as therapeutics for several reasons related to compound delivery: (i) they are commonly less than 10K molecular weight; (ii) they are more likely to be permeable to cells; (iii) they may be less susceptible to degradation by cellular mechanisms; and, (iv) they are not as apt to elicit an immune response. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, that would be desirable to screen with the assay of the present invention.

Compounds for use in the regulatable gene expression systems of the invention may be small molecules; biological or synthetic organic compounds; peptides, oligonucleotides (and derivatives thereof); or even inorganic compounds (*i.e.*, cisplatin).

Several classes of small molecules that interact with double-stranded DNA have been identified. Although the sequence binding preferences of most known DNA binding molecules have not, to date, been identified, several small DNA-binding molecules have been shown to preferentially recognize specific nucleotide sequences. In most cases, the DNA binding activity of a candidate compound is first evaluated in a pre-screening assay. In other cases, a compound with a known or predicted sequence binding preference is directly incorporated in the molecular switch system of the invention.

Preferred compounds for use in the molecular switch system of the invention include, but are not limited to dimers or multimers of known DNA-binding compounds, peptide nucleic acids (PNAs), polyamides, various triplex forming DNA-binding compounds, and derivatives thereof.

PNAs are compounds that are analogous to oligonucleotides, but differ in composition. In PNAs, the deoxyribose backbone of oligonucleotide is replaced by a peptide backbone. (See, *e.g.*, Hanvey *et al.*, 1992; Egholm, M. *et al.*, 1992; Pfeffer, N.J. *et al.*, 1993; Wittung, P. *et al.*, 1994).

Exemplary polyamides include N-methylpyrrole and N-methylimidazole amino acids which act as synthetic DNA ligands that bind to predetermined sequences in the minor

groove of DNA. (See, *e.g.*, McBryant SJ *et al.*, 1999; Bremer RE *et al.*, 1998; and White S *et al.*, 1997.)

Exemplary triplex forming DNA-binding compounds include the aromatic diamidine, DAPI (4',6-diamidino-2-phenylindole), which can induce the formation of an RNA-DNA hybrid triplex (Xu Z *et al.*, 1997); homopyrimidine PNAs which have been shown to bind complementary DNA or RNA forming (PNA)₂/DNA(RNA) triplexes (Egholm *et al.*, 1991); nucleic acid analogs such as methylphosphonates and phosphorothioates (Miller, *et al.*, U.S. Patent No. 4,757,055, issued July 19, 1988); and other small intercalating agents coupled to oligonucleotides have been described (Montenay-Garestier T., *et al.*, 1991).--

Although exemplary classes of compounds are described herein, it will be understood that any compound effective to bind to a sequence in the vicinity of the DNA response sequence for a transcriptional regulatory protein and thereby modify the binding of a transcriptional regulatory protein to its corresponding DNA response sequence finds utility in the molecular switch system of the invention.

Pre-selected compounds may be initially identified as monomers, however, such monomers may be modified or dimerized for use in the regulatable gene expression systems of the invention.

Once identified, a DNA binding compound may be modified to improve any of a number of properties, including binding affinity, transcriptional regulatory protein displacement activity, solubility, pharmacokinetics, side effects or toxicity and production cost.

Compounds for use in the molecular switch system of the invention are characterized by sequence-specific or sequence-preferential binding, binding affinity, and the ability to modify the binding of a transcriptional regulatory protein to its corresponding response element.

By way of example, a compound designated "21x" has been identified which binds to an 8 to 10 base pair stretch of AT rich double stranded DNA. 21x is a dimer of Netropsin, which is known to bind to the minor groove of DNA, and accordingly was predicted to interact with double stranded DNA through minor groove contacts.

An additional exemplary compound, GL046732, has been identified which has two linked netropsin moieties and similar binding properties to 21x.

DNA footprinting results indicate that 21x binds to the TATA box region of the IL-1 promoter region, confirming the preferential binding of 21x to AT rich sequences of DNA.

Protein displacement data indicate that when preferred 21x sequences are introduced into the DNA response sequence for UL9, NF-kB and LacR, displacement of the transcriptional regulatory protein results. (See Figs. 6, 8A-B and 10.)

In some cases, compounds which preferentially bind to "GC-rich" sequences will be used in the molecular switch systems of the invention together with any of a number of appropriate transcriptional regulatory proteins and their DNA response sequences, *e.g.*, chromomycin (Lenzmeier *et al.*, 1998; Welch *et al.*, 1994).

VI. Exemplary Systems For Regulated Gene Expression

UL9-Based Systems For Regulated Gene Expression

Chimeric transcriptional regulatory constructs containing the UL9 DNA response element were constructed. In one example, the strong sequence specific chimeric activator, UL9-VP16, was constructed with the C-terminal DNA binding domain of UL9 fused to the N-terminus of the activation domain of VP16 and expressed under the control of a CMV immediate early enhancer/promoter. Luciferase reporter constructs with a series of tandem repeated UL9 binding sites and flanking compound-binding sites were made by modifying a commercially available vector (Example 1).

When exemplary modified promoters are operably linked to the UL9 DNA response element and a reporter gene, such as firefly luciferase in a promoter test vector, *e.g.*, pGL3-basic (Promega), expression of the reporter gene may be measured in the presence or absence of a DNA binding molecule. An introduced "AT-rich" sequence results in preferential binding of a DNA binding molecule, such as 21x to the modified promoter, affecting the binding of UL9-VP16 to the UL9 DNA response element, resulting in down-regulation of transcription.

The effect of the exogenously provided chimeric activator UL9-VP16 ("ULVP") on expression of four different engineered reporter constructs in HeLa cells was evaluated. Low concentrations of pULVP encoding the UL9-VP16 activator significantly increased the expression of specific reporter constructs that have UL9 response elements while non-specific reporter constructs were not activated significantly (Example 1, Table 4). The results showed specific activation of expression by the ULVP activator promoter construct together with UL9 response elements.

The effect of an exemplary compound, 21x, on different engineered reporter constructs in MCF7 cells was also evaluated. The results suggest that reporter expression in the presence of chimeric activator ULVP was down-regulated with 21x treatment (7 fold at 20 μ M 21x) and that the observed down-regulation was concentration dependent.

Regulated Gene Expression Using A Native Transcriptional Regulatory Protein And Modifications Thereof

In one example, NF- κ B and TFIID sites of the CMV immediate early promoter are targeted with 21x or another DNA-binding compound (Example 2).

The enhancer/promoter region of the CMV immediate early promoter contains multiple cellular transcription factor binding sites, including 6x SP1, 4x CRE/ATF, 4x NF- κ B, and 2x AP1. Targeting a transcriptional regulatory protein to such DNA response elements which are modified to include compound-binding sequences may provide a means to modulate the activity of the promoter. Given that NF- κ B is implicated as an important transcription activator for the CMV promoter which is widely used in gene therapy field, oligonucleotides were constructed based on the NF- κ B DNA response sequence of the CMV promoter in order to determine if the molecular switch system described herein could be used to regulate CMV promoter the expression of genes under the control of the CMV promoter.

As detailed in Example 2, gel mobility shift assays used to detect protein displacement indicated that (1) 21x can efficiently displace p50 NF- κ B at concentrations as

low as 1 μ M, (2) the displacement is more efficient when the NF- κ B binding sequence is an IL-6 sequence (SEQ ID NO: 30) relative to an IgK sequence (SEQ ID NO:29), and (3) 21x displaces NF- κ B more efficiently than distamycin. These results suggest that the exemplary molecular switch system which utilizes 21x and NF- κ B has broad applicability to gene therapy.

The expression of exemplary modified CMV promoters operably linked to a reporter gene, such as firefly luciferase in a promoter test vector, *e.g.*, pGL3-basic (Promega) was measured in the presence and absence of the DNA binding molecule, 21x. The results show that an introduced "AT-rich" sequence resulted in preferential binding of a DNA binding molecule, such as 21x to the modified promoter, affecting the binding of NF- κ B and TFIID to the transcriptional regulatory protein DNA response element, resulting in down-regulation of transcription.

A series of purely engineered NF- κ B/ TATA binding protein (TBP) based 21x ligand switchable constructs were created having 0, 2 and 4 tandem repeats of a response element consisting of the NF- κ B response sequence flanked by 21x sites fused to a CMV minimal promoter with the TBP site modified to include a 9 A/T stretch to optimize 21x binding. These promoters were cloned into pGL3-Basic to create firefly luciferase reporter constructs, and reporter activity evaluated as detailed in Example 2.

LacR

The feasibility of using LacR as an exogenous factor for a switch-on molecular switch system was evaluated using LacR, which is a repressor that represses transcription of the lac operon by binding to lacO operator sequences. Binding and displacement of LacR was tested using oligonucleotides with introduced drug binding sites that overlap the transcriptional regulatory protein binding site (Fig. 9).

A gel mobility shift assay was carried out as described above for UL9, and the results of the assay indicate that: (1) 21x can efficiently displace LacR, and that (2) 21x appears to displace LacR more efficiently when the oligo JF107 was used, as further described in Example 3.

Regulation Of Prokaryotic Gene Expression

The *E.coli* promoter *rnb* P1 (SEQ ID NO:12), was selected as a prokaryotic model promoter for evaluating the use of 21X in the molecular switch systems of the invention, and confirming its utility in engineered switchable promoter systems.

In *Escherichia coli*, ribosome synthesis is limited by the rate of synthesis of ribosomal RNA (rRNA), which increases with growth rate. Multiple mechanisms contribute to the transcription and regulation of the *rnb* P1 promoter. These include interactions with the alpha and sigma subunits of RNA polymerase. Transcriptional control involves the UP element, and core promoter.

The (-38) to (-59) region of the promoter functions as the binding site for the α subunit of RNA polymerase (RNAP, Ross *et al.*, 1993). This AT-rich recognition element or "UP element" is responsible for the strong activity of *rnb* P1 promoter, which is 30 fold greater than activity of the promoter without the UP element. The consensus sequence of the

UP element has been previously described (Estrem *et al.*, 1998) and is shown in Fig. 2A (SEQ ID NO:13).

The *rrnB* P1 promoter UP element is composed of two sub sites, (proximal and distal), both of which are implicated in binding of the promoter to the α subunit of RNAP. The wild type UP element of *rrnB* P1, which contains a 17 base pair stretch of AT-rich sequences, was used to test the affect of various compounds which preferably bind to AT-rich sequences.

The affect of 21x on the interaction of the α subunit of RNAP with the *rrnB* P1 UP element was evaluated based on the transcriptional activity of the promoter. The sequence of nucleotides -66 to +50 of the *rrnB* P1 promoter is shown in Fig. 2B (SEQ ID NO:12).

Several *E.coli* strains carrying various *rrnB* P1 promoters fused to a *lacZ* reporter on its chromosome, were tested as a phage mono-lysogen, as detailed in Example 4.

Each of the promoters described above has intact RNAP σ binding consensus sequences in the -35 and -10 regions of the promoter.

Components of bacterial cell-based assay systems for evaluation of regulated expression using the molecular switch include:

- (1) a recombinant promoter construct including a reporter gene, such as Renilla luciferase or β -galactosidase;
- (2) a recombinant DNA response sequence which has transcription factor binding sites, such as RNA polymerase sigma and RNA polymerase alpha with drug binding sequences in the vicinity thereof; and
- (3) a small molecule (compound) designed to bind in the vicinity of the DNA response element.

In such an assay system, gene expression is measured as a function of compound concentration using wild type and engineered promoters and may include both plasmid and chromosomal DNA.

An exemplary assay is described in Example 4, below. The results indicate that the 21x effect is concentration dependent up to 10 μ M. The observed effect was not altered by targeting both sites of the UP element, relative to targeting the distal site of the UP element alone. The differences in the magnitude of the down-regulating effect of 21x suggest that the 21x binding sequence can be optimized in engineered promoters.

Such targeting studies suggest that a strong promoter like *rrnB* P1, and engineered variants thereof, can be down-regulated with a sequence preferential DNA-binding compound when the engineered promoter contains a compound binding sequence in the vicinity of the transcriptional regulatory protein DNA response element.

Regulated Gene Expression Using The Cyclin D1 Promoter

Mammalian cyclin D1 (CCND1, also named PRAD1 or BCL1) has applications to a number of cancers including but not limited to breast cancers, colon cancers and pancreatic cancers, and functions as a major positive regulator of the G₁ restriction checkpoint of the cell cycle of normal mature animal cells. (See Hunter and Pines, 1994; Sherr, 1996.)

Cyclin D1 (CCND1) is a regulatory protein overexpressed in many carcinomas. Cyclin D1 acts by binding to and regulating the cyclin dependent kinases CDK4 and CDK6.

CCND1 gene expression is low in quiescent cells (in G₀) but is induced as cells respond to growth factors and enter the cell cycle leading to an increase in active cyclin D1-CDK4/CDK6 complexes.

Rapid cell cycling irrespective of appropriate growth signals and failure to respond to growth-inhibition signals such as contact inhibition are characteristics of cancer cells.

Inappropriate expression of cyclin D1 during chromosomal inversion, translocation or amplification has been characterized in a variety of tumor cells (Hall and Peters, 1996; Sherr, 1996 for reviews). Cyclin D1 gene overexpression is also seen in many tumors without gross chromosomal rearrangements or amplification of the cyclin D1 gene. In fact, overexpression of cyclin D1 is seen in 50% of primary breast carcinomas, in 30% of adenocarcinomas of the colon (Hall and Peters, 1996), in familial adenomatous polyposis (Zhang *et al.*, 1997) as well as in many cases of pancreatic cancer (Gansauge *et al.*, 1997).

In addition, transgenic mice that overexpress the cyclin D1 gene in mammary epithelium show mammary hyperplasia and develop mammary adenocarcinomas (Wang *et al.*, 1994). Overexpression of cyclin D1 in cultured cells has been shown to result in early phosphorylation of pRB (Jiang, *et al.*, *Oncogene*, 8:3447-3457, 1993), shortening of the G1 phase and makes the cells growth factor independent (Jiang *et al.*, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). When injected into nude mice these cells produce tumors (Jiang *et al.*, 1993).

The link between inappropriate expression of cyclin D1 and tumorigenesis indicates that cyclin D1 is a good target for therapeutic intervention. Cyclin D1 antisense molecules have been shown to reduce the neoplastic phenotype of human esophageal, colon and pancreatic cancer cells overexpressing cyclin D1 in culture as well as the ability of these cells to produce tumors in mice (Zhou *et al.*, 1995; Arber *et al.*, 1997; Kornmann *et al.*, 1998). In these studies antisense technology was used to specifically inhibit cyclin D1 mRNAs.

Accordingly, regulated expression of cyclin D1 finds utility in cancer and other therapies. The present invention provides identification of DNA response elements within the cyclin D1 promoter that are involved in regulation of gene expression and a demonstration of the utility of DNA-binding compounds that bind to a sequence in the vicinity of a DNA response element of the cyclin D1 promoter as a means to modulate expression of a gene operably linked to the cyclin D1 promoter.

The human CCND1 gene has been previously cloned and sequenced (Motokura *et al.*, 1991; Withers *et al.*, 1991; Xiong *et al.*, 1991). An upstream promoter sequence of the CCND1 gene has also been cloned and sequenced (Herber *et al.*, 1994a, 1994b; Philipp *et al.*, 1994). The CCND1 promoter sequence may be found in GenBank at Accession HUMPRDA1A (Motokura and Arnold, 1993).

Potential Sp1, E2F, CRE, Oct1, Myc/Max, AP-1, Egr, NFκB, STAT5, Ets, PRAD and TCF/LEF sites have been previously identified in the cyclin D1 promoter (Motokura & Arnold 1993; Herber, Truss, *et al.* 1994; Philipp, Schneider, *et al.* 1994; Hinz, Krappmann, *et al.* 1999; Matsumura, Kitamura, *et al.* 1999; Shtutman, Zhurinsky, *et al.* 1999; and Tetsu & McCormick 1999). Several of these sites have been demonstrated to play a role in cyclin D1 regulation in various cell lines (Philipp, Schneider, *et al.* 1994; Albanese, Johnson, *et al.* 1995; Watanabe, Lee, *et al.* 1996; Yan, Nakagawa, *et al.* 1997; Watanabe, Albanese, *et al.* 1998;

Beier, Lee, *et al.* 1999; Hinz, Krappmann, *et al.* 1999; Matsumura, Kitamura, *et al.* 1999; Shtutman, Zhurinsky, *et al.* 1999; and Tetsu & McCormick 1999).

The prior art includes some analysis of the cyclin D1 promoter, but does not indicate appropriate targets for regulated gene expression using the cyclin D1 promoter. Analysis of transcription factor binding sites in the cyclin D1 promoter was carried out to identify portions of the cyclin D1 promoter that can be used to regulate the expression of a gene operably linked to the cyclin D1 promoter and important transcription factor binding sites were identified, and modified as detailed in Example 5.

A 1900-bp fragment of the human cyclin D1 promoter was PCR amplified from genomic DNA and subcloned into the vector pGL3-basic (Promega) to form a reporter construct. A series of modified promoters were made and promoter activities compared to that of the full-length (-1745) cyclin D1 promoter following transfection into asynchronous MCF7 human breast carcinoma cells, which overexpress cyclin D1, and important regulatory regions of the promoter were identified.

The -30 to -21 region of the CCND1 promoter was identified as an important regulatory region for promoter activity. The -30 to -21 sequence was modified to contain binding sites for the netropsin dimer 21x, which were introduced overlapping the -30 to -21 sequence. In one case, the site was introduced into the 3' end of the A/T-rich -30 to -21 site (SEQ ID NO:36), by changing only 2bp (10 bp 21x, SEQ ID NO:37, Example 5). A second 21x binding site was constructed by mutating 5 bp of the wild-type promoter sequence to produce an uninterrupted 8 A/T stretch (8 bp 21x, SEQ ID NO:38, Example 5). These constructs were cloned in the context of the -1745 cyclin D1 promoter in pGL3 basic, transfected into MCF7 cells and demonstrated to retain high levels of promoter activity in MCF7 cells in the absence of 21x.

Binding of 21x to these sites was confirmed using a hybridization stabilization assay, as detailed herein and described in co-owned application USSN 09/151,890 and USSN 09/393,783, incorporated herein by reference.

In summary, the binding preference of compounds to various the cyclin D1 promoter sequences was examined in a competitive hybridization-stabilization binding assay (HSA). In the HSA a nucleotide sequence of interest is represented in an oligonucleotide duplex, and the duplex was tested for its ability to compete with an indicator oligonucleotide duplex which is known to bind the test molecule with a certain degree of affinity. The indicators are rich in AT bases and labeled with either a fluorescent probe or a quencher moiety on each of the two strands. The binding of the compound to the indicator stabilizes the duplex formation allowing the fluorescence to be quenched. If the compound prefers the test sequence (competitor) more than the indicator, it is less available to stabilize the indicator duplex and thus quenching is reduced. Therefore, a higher fluorescence signal implies a higher degree of binding preference to the test sequence relative to the indicator.

In one example, the hybridization stabilization assay employs a DNA duplex as an indicator for binding, wherein one strand of the duplex is 5' labeled with fluorescein, and the complementary strand was 5' labeled with a dabsyl quenching molecule. When the two strands are mixed together with a DNA-binding molecule, which can stabilize the duplex form, the signal from the fluorescein is quenched by the dabsyl on the complementary strand. Various

cold competitor duplexes are then added to see whether they provide preferred binding sites for a DNA-binding compound, *e.g.*, 21x. If the competitor DNA, for example, an oligonucleotide containing a 21x binding site, or the wild-type cyclin D1 control sequence bind 21x, 21x is titrated away from the indicator duplex. This results in destabilization of the indicator duplex and as the strands separate, quenching is diminished and fluorescence increases.

In the experiments described in Example 5, treatment of MCF7 cells containing these constructs with 21x resulted in down regulation of cyclin D1 promoter activity while promoter constructs lacking the 21x sites were unaffected. The results show that 21x treatment of MCF7 cells was able to specifically lower cyclin D1 promoter activity 4-fold when a 21x binding site was present overlapping a transcriptional activator site.

One application of the present invention is the use of the molecular switch to modulate cyclin D1 expression in cancer cells that overexpress the gene.

Regulated Gene Expression Using the HBV core Promoter

Viral induced Hepatitis B (HBV) in humans is estimated to have infected 300 million people worldwide, with a small but significant number of infected individuals developing severe pathologic consequences, including chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma. HBV-specific promoters involved in viral replication are therefore relevant to both therapy of HBV disease and regulated gene expression which is specific to liver cells.

Characterization of the HBV core promoter, which directs the transcription of two greater than genome size messenger transcripts, has been described (for reviews, see Ganem D, in *Field Virology* 3rd Ed. 1996 and Kann M and Gerlich, W, in *Viral Hepatitis*, 2nd Ed).

The results of studies on the promoter activity of linker scanner mutants of the native sequences HBV core promoter indicated that the TATA box and proximal HNF3 sites are control elements critical for promoter activity (data not shown).

Small DNA-binding compounds were utilized to test their ability to alter the transcription level from wild type and engineered HBV core promoters, either by interference and/or displacement of protein factor binding to its cognate nucleotide binding sequences. The nucleotide composition at the core TATA box contains a run of seven A and T (adenine and thymine) bases that could serve as a preferred binding site for the compounds 21x and GL046732, which exhibit a binding preference of A/T-rich sequences. In addition, various engineered promoter constructs were prepared containing introduced A/T-rich sequences. Treatment with 21x and/or GL046732 was effective to down-regulate the core wild type promoter activity in constructs with A/T-rich sequences in a regulatory region (Example 6), indicating that DNA-binding compounds, are capable of altering levels of gene transcription through interaction with a basal transcription factor.

IX. Selection Of DNA-Binding Compounds

Exemplary pre-screening assays for candidate compounds include, but are not limited to, DNA binding assays and protein displacement assays, such as gel mobility shift assays, competitive binding assays, DNA footprinting, etc. Such assays may be carried out using various techniques which are known in the art. Briefly, an exemplary assay provides

information about the sequence-specific or sequence-preferential binding to DNA sequences, for example, binding to A/T rich sequences. Gel mobility shift assays may be used to determine the effect of a compound on the binding of a transcriptional regulatory protein to its DNA response element, based on the change in size (and corresponding mobility on a gel) of the DNA/protein complex relative to the DNA alone.

DNA footprinting may then be used to characterize the binding region based on the stability of drug binding sequence/drug complex to nuclease degradation.

In one embodiment, compounds for use in the regulatable gene expression system of the invention are pre-selected for DNA-binding and transcriptional regulatory protein displacement in a form of the Merlin™ assay. Exemplary pre-screening assays include various forms of the Merlin™ assay. See, *e.g.*, co-owned U.S. Pat. Nos. 5,306,619, 5,693,463, 5,716,780, 5,726,014, 5,744,131, 5,738,990, 5,578,444, 5,869,241, expressly incorporated reference herein.

In other embodiments, compounds are pre-selected in a nucleic acid ligand interaction assay, such as that described in co-owned, co-pending, USSN 09/151,890 (expressly incorporated by reference, herein), or another nucleic acid binding assay known to those of skill in the art.

Candidate compounds may be modified or dimerized, screened in a DNA binding and displacement assay, as further described for NF- κ B, UL9, LacR, cyclin D1 and HBV HNF3. Further evaluation of interesting compounds may then be carried out in a cell-based aspect of the molecular switch system, as further described below for UL9/VP16, *rrnB* P1 in *E. coli*, cyclin D1 and HBV HNF3 and TATA sites. The potential efficacy, toxicity and pharmacokinetic properties of a compound may be evaluated in a cellular environment in such assay systems.

In order to develop an effective regulatable *in vivo* gene expression systems, additional studies are carried out *in vivo*.

Animal models such as mice, rat, rabbit, dog, chimpanzee, zebra, fish, etc., can be employed for such *in vivo* tests.

X. In vivo Gene Therapy

A. Regulatable In vivo Expression Systems

An effective regulatable *in vivo* expression system for use in the methods and compositions of the invention must have the following properties: (1) the ability to both increase and decrease the expression of a selected therapeutic transgene, (2) the ability to tightly control the expression level of a given transgene, (3) the potential for cell type-, tissue-specific or broadly-based expression, (4) a stable vector which may be efficiently transduced into cells *in vivo* and maintain promoter activity for an extended time following transduction, (5) the ability to be regulated by a compound with minimal toxicity, (6) the ability to operate with either engineered (exogenous) or natural (native), exogenous or endogenous transcriptional regulatory elements, and (7) application to (a) treatment of genetic and non-genetic diseases (*i.e.*, cancer and infectious diseases), (b) toxic recombinant protein or secondary metabolite production, as well as (c) agricultural uses.

B. Vectors for *In vivo* Delivery of Therapeutic Genes

Successful gene therapy depends on the controlled expression of transgenes. Factors which affect the expression of such transgenes include the efficiency of transduction, the stability of the vector, and efficient activation of the promoter that regulates expression of the transgene.

The regulatable molecular switch constructs of the invention may be delivered *in vivo* by gene delivery vehicles known to those of skill in the art, including, but not limited to viral vectors (retroviral, adenoviral or adeno-associated viral vectors; Bohl, *et al.*, 1997; Bohl and Heard, 1997; Burcin, *et al.*, 1999; Ye, *et al.*, 1999) herpes virus vectors, pex virus vectors; non-viral vectors, including non-liposomal vectors (*i.e.*, FuGene™6, Roche Molecular Biochemicals), liposomal vectors (*i.e.*, DOSPER and DOTAP, Roche Molecular Biochemicals) and other non-viral means including receptor-mediated delivery, calcium phosphate transfection, electroporation, particle bombardment (gene gun), and pressure-mediated gene delivery.

In general, the efficiency of gene transfer by viral vectors, *e.g.*, retroviral vectors and adenoviral vectors, is higher than that of non-viral vectors. Retroviral vectors, including the most widely used amphotrophic murine leukemia virus (MuLV) vector, can infect only replicating cells, and typically, their transduction rate is lower than that of adenoviral vectors. However, since retroviral vectors integrate into the host genome the expression of the transgene is persistent. Recently retroviral vectors have been developed in which the therapeutic gene carrying vector construct is introduced into a packaging cell line that carries two independent constructs, which express structural proteins for packaging, thereby addressing safety issues surrounding the generation of replication competent retroviruses (Salmons and Gunzburg, 1997).

Adenoviral vectors can infect many cell types, resting and replicating, with high efficiency. However, the expression of the transgene is transient, and in addition, these vectors induce a strong host immune response. An improved adenoviral vector has the majority of the viral genome removed and increased the capacity of the vector for transgenes. Recently, a hybrid adeno/retroviral vector has been designed (Bilbao, *et al.*, 1997).

Adeno-associated virus vectors also facilitate integration of transgenes into host chromosomes, and constitutive expression of a transgene, without evoking a strong host immune response. However, limited cloning capacity, and the requirement of a helper adenovirus virus for its replication have hampered use of these types of vectors in gene therapy.

Once a transgene has been transferred into cells either via a viral or non-viral vector, expression of the transgene is governed by the strength and nature of the promoter (*i.e.*, constitutively active vs. tightly regulated). In most cases high levels of expression are preferred in the methods and compositions of the invention, and strong viral promoters are incorporated into vectors for *in vivo* expression of transgenes. However, in some cases lower levels of expression are desired, and cellular promoters are used.

Factors to be considered in order to achieve non-toxic, selective and controlled expression of transgenes include, targeted delivery of therapeutic genes to a particular tissue, cell type specific expression, and expression which may be modified by an exogenous

inducer.

For example, replicating cells may be targeted by retroviral vectors and neuronal tissue may be targeted by Herpes simplex virus (HSV) vectors. In the case of retroviral and adenoviral vectors, which lack tissue specificity, targeting may be improved, for example, by the use of recombinant pseudo-typed viruses which are produced in a packaging cell line that provides a different envelope protein (Salmons and Günzberg, 1993), by engineering the envelope protein to redirect the interaction between the envelope protein and a cell surface receptor (Valsessia-Wittman *et al.*, 1994), or to improve internalization of the vector upon receptor binding (Bushman, 1995). For adenoviral vectors, cell type specificity can be augmented by modification of the fiber protein (Wu, *et al.*, 1994). Similarly, non-viral vectors may be modified by coupling of antibodies to liposomes (Mizuno, *et al.*, 1990). In addition, incorporation of viral surface glycoproteins or fusogenic proteins into liposomes confers the tropism of the coupled molecules onto the liposomes (Morishida, *et al.*, 1993; Bagai, *et al.*, 1993).

Expression of transgenes of interest may also be controlled at the level of transcription, by the use of cell type- or developmental stage- specific promoters or promoter elements in gene transfer vectors, as further described in co-owned USSN 60/122,513, expressly incorporated by reference herein.

Although many promoters and elements confer a degree of cell type specificity, transgene expression is typically constitutive in target tissues. Temporal regulation of therapeutic transgenes is highly desirable, to avoid toxicity which may occur with constitutive expression. Promoters which are inducible by exogenous factors such as hormones, growth factors, metabolites and stress factors are useful in the methods and compositions of the invention. (See, *e.g.*, Yarranton, 1992; Gossen, *et al.*, 1993). Exemplary inducible cellular and viral promoters which exhibit restricted tissue specificity find utility in the methods and compositions of the invention, *e.g.*, the tyrosinase (Miller, *et al.*, 1995), prostate specific antigen (Culig *et al.*, 1994), α -feto protein (Ido, *et al.*, 1995) and MVMp P4 (Perros, *et al.*, 1995) promoters. Exemplary cellular promoters which are generally not tissue-specific, may also be used in the methods and compositions of the invention, *e.g.*, a glucocorticoid responsive promoter (Lu and Federoff, 1995), a heavy metal responsive promoter (Koh, *et al.*, 1995) and the cytochrome P450 1A1 promoter (Smith, *et al.*, 1995).

The feasibility of tissue-specific regulatable gene expression *in vivo* has been demonstrated by liver-specific expression using a liver-specific promoter (Burcin, *et al.*, 1999).

Gene therapy is applicable to many medical indications including monogenic diseases, multigenic diseases, oncology, infectious diseases, and acquired diseases. Temporal and spatial regulation of therapeutic transgenes is of value in many of these fields. In many of these fields molecular switch technology will be needed for optimal gene therapy protocols.

Disease targets include, but are not limited to, cancer such as prostate cancer, breast cancer, lung cancer, colorectal cancer, melanoma and leukemia; infectious diseases, such as HIV, monogenic diseases such as CF, hemophilia, phenylketonuria, ADA, familial hypercholesterolemia, and multigenic diseases, such as restenosis, ischemia, and diabetes.

In one embodiment, a natural tissue-specific promoter is modified to include one or more introduced compound binding sequences near one or more natural transcriptional regulatory factor binding sites which are essential for transcriptional regulation of the natural tissue-specific promoter.

Temporal and spatial regulation of gene expression can be achieved by combining the tissue specificity of such a promoter with regulation of the interaction between the tissue-specific promoter and one or more essential transcriptional regulatory proteins, by the exposure of the promoter to a DNA binding compound which exhibits sequence-preferential binding to the introduced compound binding sequence(s).

Once the one or more binding sites for such an essential transcriptional regulatory protein are determined, compound binding sequence(s), *e.g.* for a small molecule, are engineered into the promoter near the transcriptional regulatory protein DNA response element(s) and thereby be used to regulate the binding of the transcriptional regulatory protein to the promoter, resulting in regulation of promoter activity.

In a related aspect of the invention, a synthetic promoter is made by introducing one or more tissue-specific transcription factor binding sites and one or more compound binding sequences into the sequence of a tissue-specific regulatable promoter such that the promoter may be regulated by a compound which preferentially binds the compound binding sequence(s), *e.g.*, a small molecule. Such a small molecule may target an essential transcription factor or tissue specific transcription factor if it is essential to the activity of the promoter.

XI. EXPRESSION OF RECOMBINANT PROTEINS

In vitro

Suitable host cells for cloning or expressing recombinant proteins include prokaryotic, yeast, and higher eukaryotic cells. Suitable prokaryotes include, but are not limited to, gram-negative and gram-positive bacteria, for example, *E. coli*, various strains of which are publicly available.

Host cells are transfected or transformed with expression or cloning vectors for recombinant protein production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, and/or amplifying the expression of genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, may be optimized according to knowledge generally available to those of skill in the art. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Butler, 1991, and Sambrook, *et al.* 1989.

Methods of transfection are known to those of skill in the art, for example, CaPO_4 transfection, bacterial protoplast fusion with intact cells, nuclear microinjection, electroporation, or in methods that employ polycations, such as, polybrene or polyornithine. Transfection is carried using standard techniques, as appropriate to the particular type of cells being transformed.

Infection with *Agrobacterium tumefaciens* is generally used for transformation of plant cells, as described by Shaw, *et al.*, 1983 and WO 89/05859 published 29 June 1989.

Mammalian cell transformations may be carried out as generally described in U.S. Patent No. 4,399,216: Keown, *et al.*, 1990 and Mansour, *et al.*, 1988.

In addition to prokaryotes, eukaryotes such as filamentous fungi or yeast are useful for expression of recombinant proteins. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism.

Expression of recombinant proteins in yeast are typically carried out following transfection according to the methods described in Van Solingen, *et al.*, 1977 and Hsiao, *et al.*, 1979.

Suitable host cells for the expression of glycosylated recombinant proteins are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line, 293, Graham, *et al.*, (1977); Chinese hamster ovary cells (Cho, *et al.*, (1980); human lung cells (W138, ATCC CCL 75); and human liver cells (Hep G2, HB 8065). Large numbers of cell lines are publicly available, *e.g.*, from the American Type Culture Collection (ATCC, Manassas, VA). The selection of the appropriate host cell is deemed to be within the skill in the art.

In general, in methods for production of recombinant proteins, the nucleic acid (*e.g.*, cDNA or genomic DNA) encoding a recombinant protein or polypeptide of interest is inserted into a replicable vector for cloning, or for expression. Various vectors are publicly available, and may take the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid coding sequence may be inserted into the vector by a variety of procedures known to those skilled in the art of recombinant DNA technology.

In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The desired recombinant protein or polypeptide may be produced recombinantly directly, or as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. Included in heterologous nucleic acid constructs for use in the methods of the invention are signal sequences that allow processing and translocation of the protein, as appropriate. The heterologous nucleic acid construct typically lacks any sequence that might result in the binding of the desired protein to a membrane.

In some cases, the recombinant protein may be produced as a precursor protein, which may be further processed in cell culture or following extraction from the culture medium.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is

suitable for most gram-negative bacteria, and various viral origins of replication (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

In cases where two separate plasmids are transformed into bacteria, compatible replicons are used employing techniques generally known to those of skill in the art.

5 In most cases, expression and cloning vectors also contain a selectable marker gene. Typical selectable marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

10 Expression and cloning vectors generally contain a promoter operably linked to the recombinant protein- or polypeptide-encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Such promoters may be inducible or constitutive, and may be of prokaryotic, eukaryotic or viral origin.

15 In the methods and compositions of the invention, the molecular switch systems described herein are used for expression of recombinant proteins and polypeptides.

20 When an endogenous transcriptional regulatory protein is utilized in the molecular switch system of the invention, a vector is provided which includes a DNA binding site for the transcriptional regulatory protein, a compound-binding sequence, a promoter, and a transgene which encodes a recombinant protein or polypeptide of interest, under the control of the aforementioned promoter.

25 In some cases, the molecular switch systems of the invention for expression of recombinant proteins include two vectors, wherein one vector comprises the DNA binding site for a transcriptional regulatory protein, a compound-binding sequence, a first promoter, and a transgene which encodes a recombinant protein or polypeptide of interest, under the control of the aforementioned promoter. A second vector is effective to express an engineered transcriptional regulatory protein or natural regulatory protein having a regulatory domain and a DNA binding domain under the control of a first promoter (inducible or constitutive). The regulatable expression system also includes compounds or inducers which
30 bind to the compound-binding sequence.

35 In other cases, a single vector system is used for expression of recombinant proteins *in vitro*. In such cases, the vector includes the DNA binding site for a transcriptional regulatory protein, a compound-binding sequence, a first promoter, and a transgene which encodes a recombinant protein or polypeptide of interest, under the control of the first promoter and is effective to express an engineered transcriptional regulatory protein or natural regulatory protein under the control of a second promoter. The expression of one or both of the transgene and transcriptional regulatory protein may be under the control of a constitutive or compound-inducible promoter.

40 In still other cases, a single vector is effective to express both a transcriptional regulatory protein and a transgene under the control of a single compound-inducible promoter, utilizing internal ribosomal entry sites (IRES).

Alternatively, the molecular switch comprises a single vector which has a transcriptional regulatory protein under the control of a single compound-inducible and a

transgene under the control of a constitutive promoter.

Transcription of a DNA encoding a recombinant protein or polypeptide by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually from about 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes, however, frequently eukaryotic viral enhancers are used. The enhancer may be incorporated into the vector at a position 5' or 3' to the recombinant protein or polypeptide coding sequence, but is preferably located at a site 5' to the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, or human) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 3' and, occasionally 5', untranslated regions of eukaryotic or viral DNAs or cDNAs.

Molecular biological procedures routinely employed by those of skill in the art for production of recombinant proteins are provided in Sambrook, *et al.*, 1989 and Ausubel, *et al.*, 1989, both of which are expressly incorporated by reference herein.

Heterologous nucleic acid constructs for use in the methods of the invention may encode any protein or polypeptide of interest, or an intermediate in a biosynthetic pathway leading to a product or secondary metabolite of interest.

Exemplary recombinant proteins or polypeptides which may be expressed using the molecular switch systems of the invention, include, but are not limited to, enzymes; immunoglobulins; recombinant proteins such as those used in therapeutics; including, but not limited to; serum albumin; Factor VIII, tissue plasminogen factor, erythropoietin, colony stimulating factors, such as G-CSF and GM-CSF, cytokines such as interleukins, integrins; surface membrane protein receptors; T cell receptors; structural proteins, such as, collagen, fibrin, elastin, tubulin, actin, and myosin; growth factors and growth hormones. The protein may also be an industrial protein or enzyme as exemplified by peroxidase, glucanase, alpha-amylase, and glucose oxidase).

Such exemplary recombinant proteins or polypeptides may be expressed using the molecular switch systems of the invention in the context of *in vitro* expression in bacteria, yeast, insect cells, mammalian cells and plant cells as well as *in vivo* in transgenic animals and plants.

In one further embodiment the molecular switch system may be used to express more than one recombinant protein at the same time. For example, a "switch on" system using a transcriptional regulatory protein with a repressor as the regulator component could be used to increase expression of one recombinant protein at the same time a "switch off" system using a transcriptional regulatory protein with an activator component is used to decrease expression of a second protein, *e.g.*, a proteolytic enzyme.

In vivo in Transgenic Animals

Nucleic acids which encode recombinant proteins, polypeptides, and modified forms thereof, may be used to generate transgenic animals which, in turn, are useful in the production of therapeutically useful reagents. A transgenic animal (*e.g.*, a mouse, rat or goat) is an animal having cells that contain a transgene, which transgene was introduced into

the animal or an ancestor of the animal at a prenatal, *e.g.*, an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding a polypeptide or protein of interest can be used to clone genomic DNA encoding that polypeptide or protein in accordance with established techniques. Methods for generating transgenic animals, particularly animals such as mice, rats and goats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866, 4,870,009 and 5,907,080.

Typically, transgenic animals that include a copy of a transgene encoding a polypeptide or protein of interest introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding the polypeptide or protein of interest.

Recently, transgenic animals are being used to produce various types of recombinant proteins. Transgenic goats which produce therapeutic proteins in their milk have been developed and recently a commercial kit, the pBC1 Milk Expression Vector Kit (Genzyme Transgenics Corporation and Invitrogen Corp.), became available for the production of recombinant proteins in the milk of transgenic mice. In such methods, the DNA sequences for a milk protein promoter is operably linked to the coding sequence for a recombinant protein or polypeptide of interest. Similarly, the molecular switch system described herein find utility in regulated, *e.g.*, switch-on, expression of recombinant proteins or polypeptides of interest in transgenic animals.

XII. Agricultural Applications

A. Regulation of Gene Expression

Regulatable gene expression is applicable to many agricultural uses as well. This aspect of the invention includes methods directed to the production of transgenic plants using the regulatable expression (molecular switch) systems of the invention, resulting in the production of; (1) non-native recombinant proteins and polypeptides, (2) modified native proteins and polypeptides, and (3) secondary metabolites in such transgenic plants.

Regulation of transcription using exogenous bacterial transcriptional repressors such as LacR and TetR together with plant promoters modified to contain an appropriate bacterial operator sequence, have been successfully employed in various plant systems such as Arabidopsis, carrot and tobacco cells (Gatz, *et al.*, 1991; Wilde, *et al.*, 1992; Ulmasov, *et al.*, 1997).

The use of chimeric transcriptional activators such as LacR/Gal4 (Moore *et al.*, 1998) and Gal4/VP16 or Gal4/THM18 (Schwechheimer, *et al.*, 1998) for the regulation of transgene expression from engineered promoters has also been demonstrated in plant systems.

The molecular switch system of the invention finds utility in the regulation of plant gene expression by providing either an exogenous or endogenous transcriptional regulatory factor (repressor or activator), which is active in plants, together with a corresponding DNA response element for the transcriptional regulatory factor, a compound binding site and a DNA-binding compound which preferentially binds to the compound binding site.

In most cases, gene expression is achieved by introducing a single vector or nucleic

acid construct into plant cells, wherein the vector includes either: (1) a DNA response element for a transcriptional regulatory protein, a compound-binding sequence, a promoter, and a transgene which encodes a recombinant protein or polypeptide of interest, under the control of the promoter, which functions together with a native transcriptional regulatory protein and an exogenously supplied DNA binding compound or (2) a DNA response element for a transcriptional regulatory protein, a compound-binding sequence, a promoter, and a transgene which encodes a recombinant protein or polypeptide of interest, under the control of the promoter, together with an engineered transcriptional regulatory protein or natural regulatory protein also under the control of a promoter, which functions together with an exogenously supplied DNA binding compound.

In some cases, gene expression is achieved by introducing two vectors or nucleic acid constructs into plant cells, wherein a first vector is effective to express an engineered transcriptional regulatory protein or natural regulatory protein, and a second vector includes a DNA binding sequence for the transcriptional regulatory protein, a compound-binding sequence, a promoter, and a transgene which encodes a protein or polypeptide of interest, under the control of the aforementioned promoter, which function together with an exogenously supplied DNA binding compound.

Both the one and two vector aspects, and the one and two promoter aspects of the molecular switch system of the invention include compounds or inducers which bind the compound-binding sequence. Exemplary compounds for use in the molecular switch system of the invention are further described above.

B. Exemplary Plant Transcription Factors and Associated Binding Proteins

Exemplary transcriptional regulatory factors for use in plants include the UL9/VP16 activator or UL9/KRAB repressor, together with a regulatable transgene operably linked to a promoter having one or more UL9 DNA response elements in the vicinity of one or more binding sequences for 21x.

It will be understood that the various components of the molecular switch system are interchangeable. For example, transcriptional regulatory factors for use in the methods of the invention may include any of a number of DNA binding domains, such as DAT1 from *Saccharomyces cerevisiae*. DAT1 specifically recognizes the minor groove of non-alternating oligo(A).oligo(T) sequences (Reardon, *et al.*, 1995), and accordingly provides a sequence for the effective binding of 21x and compounds which act by a similar mechanism.

In one example, a heterologous nucleic acid construct is described which has the coding sequence for a reporter or gene of interest, linked to a minimal promoter (*i.e.* CaMV 35S) with two upstream lac operator sequences fused to the promoter sequence, which serve as the binding site for a transcription factor, "LhG4". LhG4 has a transcriptional activator domain from Gal4 fused to a mutant *lac*-repressor, which has enhanced binding affinity, and functions to regulate transcription of coding sequences downstream of the CaMV 35S promoter. (See, *e.g.*, Moore, *et al.*, 1998).

The tet repressor-operator system has been used to regulate the gene expression in transgenic tobacco plants. A transgenic plant constitutively synthesizing a large number of Tet repressor monomers per cell was made, followed by introduction of a heterologous

nucleic acid construct containing the beta-glucuronidase (Gus) gene under the control of a CaMV 35S promoter, modified to contain two tet operators. Expression of the GUS gene was repressed 50- to 80-fold when both operators were positioned downstream of the TATA box. (See, *e.g.*, Gatz, *et al.*, 1991).

In some cases, the molecular switch system may make use of endogenous transcription factors found in plants. For example, the endogenous plant transcriptional activator 780BP (780 binding protein) of cauliflower inflorescence which binds to the 780 gene of T-DNA may be used. The DNA response element was determined (Adams and Gurley, 1994; TTGAAAAATCAACGCT, SEQ ID NO:23) and includes the preferred sequence for binding of 21x and other compounds which target "AT-rich" sequences.

In one exemplary embodiment, tandem repeats of the 780BP DNA response element are fused to the minimal CaMV 35S promoter sequence operably linked to a transgene, and 21x is used to regulate the binding of 780BP at the tandem repeated sites.

In a further exemplary embodiment, a plant tissue-specific transcription factor, NtBBF1, identified by its ability to bind to a regulatory domain of the *rolB* oncogene promoter (found in the *Agrobacterium rhizogenes* Ti plasmid in tobacco), is used to regulate transcription. The DNA response (cis) element for NtBBF1 has been identified in the *rolB* gene (ACTTTA, SEQ ID NO:27). Mutational studies have indicated that this sequence is essential for the expression of *rolB* in apical meristems (Baumann, *et al.*, 1999). A tissue specific regulatable promoter may be designed using the DNA response element for NtBBF1 in the *rolB* promoter or an engineered promoter having the DNA response element for NtBBF1 fused to a minimal promoter sequence wherein the sequence in the vicinity of the DNA response (cis) element for NtBBF1 is modified to include small molecule binding sequences (*i.e.*, 21x). For example, the NtBBF1 *cis* element (bold, uppercase), may be modified to include one or more introduced compound binding sequences (lowercase) for 21x or another compound that preferentially binds to "AT-rich" sequences. Potential compound binding sequences are indicated as "()".

AC(TTTAtttt)
(aaaACTTTA)

The DNA response element for NtBBF1 may be fused to a minimal promoter in tandem to increase the activity of the promoter.

Overexpression of the natural plant transcription factor, "CBF1", which binds to a DNA response element, "CRT/DRE", found in the promoter of cold-inducible genes may find utility in regulating cold tolerance by incorporating CBF1 and CRT/DRE into the molecular switch systems of the invention. (See, *e.g.*, Warren, 1998).

A cis-acting element identified in the promoter region of the *rd29A* gene is associated with dehydration and cold-induced gene expression. The sequence designated the dehydration response element ("DRE", TACCGACAT, SEQ ID NO:28), has been found in the promoter regions of other dehydration and cold-stress inducible genes. When the stress inducible promoter *rd29A* was used to drive expression of a DRE-binding protein, "DREB1A" in *Arabidopsis*, transgenic plants were produced that were drought-, salt- and

freezing-tolerant. (Kasuga, *et al.*, 1999). The DREB1A transcriptional regulatory protein and the DRE response element, may find utility in regulating drought-, salt- and cold-tolerance by incorporating them into the molecular switch systems of the invention.

5 Plant output traits of interest may be modified using the methods of the invention by introducing heterologous nucleic acid constructs which encode recombinant proteins, polypeptides, or intermediates in the biosynthetic pathway leading to the production of metabolites associated with such output traits.

Such heterologous nucleic acid constructs may encode native or non-native, *e.g.*, mammalian or viral proteins or polypeptides.

10 In another aspect of the invention, recombinant proteins or polypeptides are produced in plants using the molecular switch methods of the invention.

C. Improved Output Traits

15 The development of plants having desired traits such as improved yield; disease resistance to fungal, bacterial, viral and other pathogens; insect resistance; herbicide resistance; improved fruit ripening characteristics; cold temperature and dehydration tolerance; increased salt and drought tolerance; improved food quality (*i.e.* nutritional content) and improved appearance has been the focus of agribusiness for many years.

20 Numerous genes involved in regulating such plant characteristics have been identified and characterized

One example is the development of herbicide resistance in rice plants. Transformed rice has been shown to be resistant to at least imazethapyr, imazaquin, nicosulfuron, and primisulfuron, with suggested resistance to additional herbicides. (See, *e.g.*, U. S. Pat. No. 5,773,703.)

25 Another example is genetically altered higher plants having a modified starch and sucrose biosynthesis phenotype, *e.g.*, edible plants, such as peas with altered sucrose and starch content. (See, *e.g.*, U. S. Pat. No. 5,773,693.)

30 Coding sequences for expression in plants using the regulatable expression vectors described herein include, but are not limited to, sequences which encode enzymes and other proteins or polypeptides that confer: disease resistance to fungal, bacterial, viral and other pathogens; insect resistance; herbicide resistance; fungicide resistance; and insecticide resistance.

35 Coding sequences associated with output traits of interest further include, those associated with: regulation of plant development; regulation of fruit ripening; increased salt and drought tolerance; and regulation of plant nutritional content, *e.g.*, by altered oil composition in seeds, increased grain oil content, altered seed protein composition, altered carbohydrate composition in seeds, altered carbohydrate composition in fruits, and the like. (See, *e.g.*, Brar, *et al.*, 1996).

40 By way of example, numerous plant proteins associated with pathogenesis or pathogenesis-related proteins (PR proteins) which are induced in large amounts in response to infection by various pathogens, including viruses, bacteria and fungi have been identified.

In one aspect of the invention, the use of heterologous nucleic acid construct comprising the coding sequence for such pathogenesis-associated proteins can be used in the

molecular switch systems of the invention to develop plants which have enhanced resistance to disease. (See, *e.g.*, Redolfi, *et al.*, 1983; Van Loon, 1985; and Uknes, *et al.*, 1982; and U. S. Pat. No 5,880,328, issued Mar. 9, 1999.)

5 D. Production of Recombinant Proteins and Polypeptides in Plants

Transgenic plants as the source of recombinant proteins and polypeptides offer the advantage of production at low cost, based on ease of plant transformation and scale up, correct assembly of the subunit components of multimeric proteins, and the lack of pathogens associated with recombinant protein or polypeptide production in cell culture. (See, *e.g.*,
10 Larrick, 1998).

Heterologous nucleic acid constructs for use in the methods of the invention may include coding sequences for recombinant proteins or polypeptides for pharmaceutical applications and nutraceutical production.

Exemplary recombinant proteins which have been produced in plants include
15 vaccines, enzymes, hormones, plasma proteins, and antibodies. More recently technology has been developed for the production of polymers, such as microbial polyesters in plants. (See, *e.g.*, Kolodziejczyk, 1999).

More specific examples of recombinant proteins which have been produced in plants include, SpaA of *S. mutans*, HBV surface antigen, M protein of HBV, LT of *E. coli*, CT of
20 *V. cholerae*, capsid protein of Norwalk virus, rabies glycoprotein, VP1 of foot and mouth disease virus, secretory IgA and IgG. (See, *e.g.*, Ma and Vine, 1999; Tian and Yang, 1998; Larrick, 1998).

 E. Plant Transformation

25 Genetic transformation of plants is generally accomplished by introducing heterologous nucleic acid constructs into plants using *Agrobacterium* T-DNA vectors, microprojectile bombardment or by use of plant viral vectors, including, but not limited to, tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), tomato bushy stunt virus and alfalfa mosaic virus (AIMV), potato virus X (PVX) (Ma and Vine, 1999; Smolenska, *et al.*,
30 1998).

Targeting recombinant proteins for secretion in plants may be accomplished using either native or plant-derived leader sequences, such that N-glycosylation takes place. The expression of recombinant proteins or polypeptides may be targeted to extracellular spaces or to particular tissues, *e.g.*, storage organs such as seeds, by use of tissue-specific promoters.

35 Once expressed, such recombinant proteins or polypeptides may be extracted and purified using techniques generally available to those of skill in the art. Optimal methods of plant transformation vary dependent upon the type of plant. It is preferred that the vector sequences be stably integrated into the plant genome.

Preferred methods for transformation of plant cells in molecular switch methods of
40 the invention are *Agrobacterium*-mediated transformation, electroporation, microinjection, and microprojectile bombardment.

In another aspect of the invention, transgenic plants are produced following infection with a plant virus which has been genetically modified to encode one or more foreign genes,

which are expressed following infection, as a soluble protein or polypeptide in the plant cytoplasm, targeted to cellular compartments, or alternatively fused to a viral coat protein which is displayed on the surface of the viral particle.

Expression vectors for use in the molecular switch methods of the invention comprise heterologous nucleic acid constructs, designed for operation in plants, with companion sequences upstream and downstream from the expression cassette. The companion sequences are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the plant host, such as, sequences containing an origin of replication and a selectable marker. Typical secondary hosts include bacteria and yeast.

In one embodiment, the secondary host is *E. coli*, the origin of replication is a colE1-type, and the selectable marker is a gene encoding ampicillin resistance. Such sequences are well known in the art and are commercially available as well (e.g., Clontech, Palo Alto, Calif.; Stratagene, La Jolla, CA).

Vectors useful in the practice of the present invention may be microinjected directly into plant cells by use of micropipettes to mechanically transfer the nucleic acid construct or cassette (Crossway, Mol. Gen. Genet, 202:179-185, 1985). Such nucleic acid constructs or cassettes may also be transferred into the plant cell using polyethylene glycol (Krens, *et al.*, 1982).

High velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface may also be used for introduction of nucleic acid sequences into plant cells. (See, e.g., Klein, *et al.*, 1987 and Knudsen and Muller, 1991).

Yet another method for introduction of nucleic acid sequences into plant cells is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible for introduction of nucleic acid segments into plant cells with lipid surfaces (Fraley, *et al.*, 1982).

A preferred method for introduction of nucleic acid constructs or cassettes into the plant cells is electroporation (From, *et al.*, 1985). In this technique, electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of plasmids into plant cells or protoplasts. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.

Another preferred method of introducing a nucleic acid construct comprising a sequence of interest into plant cells is to infect a plant cell, explant, meristem or seed with *Agrobacterium*, in particular *Agrobacterium tumefaciens*. A nucleic acid construct comprising such a sequence of interest can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome (Horsch, *et al.*, 1984; Fraley, *et al.*, 1983; Schell, 1987).

Standard *Agrobacterium* binary vectors are known to those of skill in the art and many are commercially available. Expression vectors typically include polyadenylation sites, translation regulatory sequences (e.g., translation start sites), introns and splice sites, enhancer sequences (which can be inducible, tissue specific or constitutive), and may further include 5' and 3' regulatory and flanking sequences.

An exemplary binary vector suitable for use in the molecular switch methods of the invention include at least one T-DNA border sequence (left, right or both); restriction endonuclease sites for the addition of one or more heterologous nucleic acid coding sequences [adjacent flanking T-DNA border sequence(s)]; a heterologous nucleic acid coding sequence (*i.e.*, the sequence encoding a protein or polypeptide of interest), operably linked to appropriate regulatory sequences and to the directional T-DNA border sequences; a selectable marker-encoding nucleotide sequence which is functional in plant cells, operably linked to a promoter effective to express the selectable marker encoding sequence; a termination element for the selectable marker-encoding nucleotide sequence; a heterologous Ti-plasmid promoter; a nucleic acid sequence which facilitates replication in a secondary host (*e.g.*, an *E. coli* origin of replication) and a nucleic acid sequence for selection in the secondary host, *i.e.*, *E. coli*.

In general, a selected nucleic acid sequence is inserted into an appropriate restriction endonuclease site or sites in the vector. Standard methods for cutting, ligating and *E. coli* transformation, known to those of skill in the art, are used in constructing vectors for use in the present invention. See, for example, Sambrook, *et al.* (1989) and Ausubel, *et al.*, (1989).

In choosing a promoter it may be desirable to use a tissue-specific or developmentally regulated promoter for regulated expression in certain tissues without affecting expression in other tissues. Numerous examples of such promoters are known in the art or differential screening techniques may be used to isolate promoters expressed at specific (developmental) times, such as during seed development.

Generally, the construction of vectors for use in practicing the present invention are known by those of skill in the art. (See generally, Maniatis, *et al.*, (1989), and Ausubel, *et al.*, (c) 1987, 1988, 1989, 1990, 1993 by Current Protocols; Gelvin, *et al.*, (1990), all three of which are expressly incorporated by reference, herein.

In one aspect of the invention, an *Agrobacterium* binary plant transformation vector is introduced into a disarmed strain of *A. tumefaciens* by electroporation (Nagel, *et al.*, 1990), followed by co-cultivation with plant cells, to transfer the heterologous nucleic acid construct(s) into plant cells. Upon infection by *Agrobacterium tumefaciens*, the heterologous DNA sequence is stably integrated into the plant genome in one or more locations.

In a further aspect of the invention, transgenic plants are produced using *Agrobacterium* T-DNA vectors or microprojectile bombardment, where a heterologous nucleic acid coding sequence is integrated into the plant genome and traditional breeding is used to generate transgenic seed stock and transgenic plants.

In a further aspect, plant cells are transformed by infection with *Agrobacterium tumefaciens*. However, as will be appreciated, the optimal transformation method and tissue for transformation will vary depending upon the type of plant being transformed.

Suitable selectable markers for selection in plant cells include, but are not limited to, antibiotic resistance genes, such as, kanamycin (*nptII*), G418, bleomycin, hygromycin, chloramphenicol, ampicillin, tetracycline, and the like. Additional selectable markers include a bar gene which codes for bialaphos resistance; a mutant EPSP synthase gene which encodes glyphosate resistance; a nitrilase gene which confers resistance to bromoxynil; a mutant

acetolactate synthase gene (ALS) which confers imidazolinone or sulphonylurea resistance; and a methotrexate resistant DHFR gene.

The particular marker gene employed is one which allows for selection of transformed cells as compared to cells lacking the DNA which has been introduced.

5 Preferably, the selectable marker gene is one which facilitates selection at the tissue culture stage of the molecular switch methods of the invention, *e.g.*, a kanamycin, hygromycin or ampicillin resistance gene.

10 Transformed explant cells are screened for the ability to be cultured in selective media having a threshold concentration of selective agent. Explants that can grow on the selective media are typically transferred to a fresh supply of the same media and cultured again. The explants are then cultured under regeneration conditions to produce regenerated plant shoots. After shoots form, the shoots are transferred to a selective rooting medium to provide a complete plantlet. The plantlet may then be grown to provide seed, cuttings, or the like for propagating the transformed plants. The method provides for high efficiency
15 transformation of plant cells with expression of modified native or non-native plant genes and regeneration of transgenic plants, which can produce a protein, polypeptide or secondary metabolite of interest.

Once the expression of a protein, polypeptide or secondary metabolite of interest is confirmed using standard analytical techniques such as Western blot, ELISA, PCR, HPLC, NMR, or mass spectroscopy, whole plants are regenerated. Plant regeneration is described
20 for example in Evans, *et al.*, 1983 and in Vasil, 1984, and 1986).

XIII. Utility Of The Invention

The present invention can be used for (1) screening and optimizing, as well as validation
25 of the sequence specificity of a DNA binding molecule in cell based assays, (2) in vectors for controlled therapeutic gene expression *in vivo*, (3) in toxic protein production in eukaryotic expression systems, (4) for recombinant protein and secondary metabolite production, (5) in various agricultural uses, examples of which are described above, (6) as a research tool, and (7) in developmental and functional studies with transgenic animals, where molecular switches
30 allow the temporal expression of the genes that are lethal if expressed at an early stage of development. Expression of disease or therapeutic genes in adult animals may aid the study of the function of these genes.

IX. Advantages

35 All of the prior art systems for regulated gene expression rely on the binding of a compound to a regulatory protein and each lacks some features of an effective regulatable expression system.

The molecular switch compositions and methods described herein provide the advantage of regulated gene expression using native transcriptional regulatory proteins which
40 are present endogenously and which may also be exogenously provided.

In contrast to the prior art, in the molecular switch methods and compositions of the invention, the compound binds with double-stranded DNA and the binding of the compound to double-stranded DNA has an effect on the binding of a transcriptional regulatory protein to its

DNA response element. In the methods of the invention, any compound which modulates the binding of a transcriptional regulatory protein to its DNA binding site can be used to regulate the expression of a gene operably linked to the promoter. The choice of inducer is not restricted by the transcriptional regulatory protein as long as it modifies the binding of the transcriptional regulatory protein to its DNA response element and thereby regulates the expression of a gene operably linked thereto.

By engineering one or more compound binding sequences in the vicinity of the DNA response element for an endogenous transcriptional regulatory protein, a compound can specifically target transcription factor binding to the engineered site or sites, resulting in greater specificity of regulation.

In addition, the invention provides a system that is tightly regulated by an exogenous factor which can regulate expression of the transgene without non-specifically affecting expression of endogenous cellular genes.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

EXAMPLE 1

UL9 Chimeric Transcriptional Regulatory Constructs

Oligonucleotides comprising the UL9 DNA response element and one or two binding sequences for the A/T-rich binder, 21x were constructed. In each oligonucleotide the putative 21x-binding sequence(s) overlap the modified UL9 binding site (SEQ ID NO:18). The modified sequences include YK 202LX (Fig. 6, SEQ ID NO:19), YK 202RX-A (Fig. 6, SEQ ID NO:20), and YK 202RX (Fig. 6, SEQ ID NO:21), wherein the transcriptional regulatory protein DNA response site is indicated as bolded and uppercase, introduced compound binding sequences are indicated in lowercase and potential compound binding sequences are indicated as () or []. A gel mobility shift assay for protein displacement was used to measure compound induced protein displacement. A ³²P labeled oligonucleotide was incubated with 10 nM GST-UL9 at room temperature in the binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 5% glycerol and 1mM DTT) for 20 minutes, followed by the addition of 21X. The incubation was continued for 2 hours and the samples analyzed by polyacrylamide gel electrophoresis, with the amount of protein bound oligonucleotide quantitated. UL9 was displaced most efficiently when there was an overlap between protein and 21x binding sequences at 3'end of UL9 binding site, as shown in Fig. 7.

UL9 Activator Constructs

The strong sequence specific chimeric activator, UL9-VP16, was constructed the C-terminal DNA binding domain of UL9 fused to the N-terminus of the activation domain of VP16 utilizing pGEX-UL9 (Genelabs) and pACT (Promega), expressed under the control of a CMV immediate early enhancer/promoter. Luciferase reporter constructs with a series of

tandem repeated UL9 binding sites and flanking compound-binding sites were made by modifying the pG5luc vector (Promega). In this vector the fire fly luciferase is under the control of synthetic promoter that is composed of five tandem repeats of GAL4 binding sites followed by the major late minimal promoter of adenovirus. Gal4 binding sites in the vector were replaced with 1 to 7 copies of the UL9 binding site.

The effect of the exogenously provided chimeric activator UL9-VP16 ("ULVP") on expression of four different engineered reporter constructs was evaluated. p5UL and p5ULE were engineered with the adeno major late minimal promoter fused to 5 tandem repeats of the UL9-21x response element and a firefly luciferase reporter in the pGL3-Basic or the pGL3-Enhancer vector which has an SV40 enhancer, respectively. pULVP has a chimeric UL9/VP activator fused to a firefly luciferase reporter. p5Gal and p5GalE contain 5 tandem repeats of the Gal4 response element in place of the UL9-21x response element of p5UL and p5ULE, respectively. The promoterless pRL-Null plasmid containing the Renilla luciferase reporter was used as a copy number control.

HeLa cells (5×10^5 cells) were co-transfected with 3 plasmids: 2 μ g of reporter, 0.2 μ g of pRL-Null co-reporter and varying amounts of pULVP (0 to 100 ng). Low concentrations of pULVP encoding the UL9-VP16 activator significantly increased the expression of specific reporter constructs that have UL9 response elements while non-specific reporter constructs were not activated significantly (Table 4). P5UL and p5ULE expression was increased 24 fold and 8 fold, respectively above basal expression, with 25 ng of pULVP. In contrast, 25ng of pULVP activated p5Gal only 2 fold and did not activate p5GalE expression at all. SV40 enhancer in p5ULE and p5GalE augmented the promoter activities 18 fold and 15-fold compared to the activities of comparable constructs with no enhancer (p5UL and p5Gal), respectively.

Table 4 Effect Of UL9-VP16 Activator On Reporter Expression

<u>Construct</u>	<u>no pULVP</u>	<u>pULVP (25ng)</u>	<u>pULVP</u>
p5UL	1 x	24 x	31 x (1 ng), 77 x (20 ng)
p5ULE	18 x	138 x	ND ¹
p5Gal	1 x	2 x	ND
p5GalE	15 x	17 x	ND
pRL-Null	1 x	1.5~2.5 x	3 x (10 ng)

The results indicate that exogenously provided ULVP acts as a transcriptional activator for promoters which have UL9 response elements. Further titration (0 to 40 ng) of pULVP was carried out to determine the optimal level of ULVP for the specific activation of p5UL and p5ULE. Based on firefly luciferase expression normalized by Renilla luciferase expression from pRL-Null, 1 ng of pULVP showed an activation level relative to p5ULE of over 30 fold. Expression of ULVP also increased expression of pRL-Null up to 3 fold increase was observed with 10 ng of pULVP. The non-normalized reporter activity indicated

¹ ND=not done

up to 77-fold activation of p5ULE with 20 ng of pULVP (Table 4).

The results show specific activation of expression by the ULVP activator promoter construct together with UL9 response elements.

5x10⁴ MCF7 cells were co-transfected with 3 µg of reporter, 0.5 µg of pRL-Null co-reporter and 20ng of pULVP using 16 µg of LipofectAmine™ and 2 µl of Plus agent in a total volume of 0.4 ml in each well of a 24-well plate (1 % fetal calf serum OPTI-DMEM medium). After 4 hours medium was changed to OPTI-DMEM containing 1% fetal calf serum plus varying amount of 21x. 20 ng of pULVP activator was shown to significantly increase the expression of p5UL which has UL9 response elements while the control reporter construct p5Gal was not activated significantly (Figure 11). p5UL reporter expression in the presence of chimeric activator ULVP was down-regulated significantly with 21x treatment (7 fold at 20 µM 21x). The down-regulation was concentration dependent, suggesting that 21x displaced the ULVP chimeric activator from the promoter and that the 21x ligand response element was UL9 specific.

UL9 Repressor Construct

The sequence specific chimeric repressor, UL9-KRAB, was constructed the C-terminal DNA binding domain of UL9 fused to the N-terminus of the repressor domain of kruppel protein (KRAB, SEQ ID NO:10, Margolin JF, *et al.*, 1994), expressed under the control of a CMV immediate early enhancer/promoter. Luciferase reporter constructs with a series of tandem repeated UL9 binding sites and flanking compound-binding sites were made by modifying the pG5luc vector (Promega). In this vector the firefly luciferase is under the control of synthetic promoter that is composed of five tandem repeated GAL4 binding sites followed by the major late minimal promoter of adenovirus. Gal4 binding sites in the vector were replaced with 1 to 7 copies of the UL9 binding site.

The effect of the exogenously provided chimeric repressor UL9-KRAB ("ULKRAB") on expression of three different engineered reporter constructs was evaluated. p5ULE was engineered with the major late minimal promoter of adenovirus fused to 5 tandem repeats of the UL9-21x response element and a firefly luciferase reporter in the pGL3-Enhancer vector which has an SV40 enhancer. p5GalE has five tandem repeats of the GAL4 binding site followed by the major late minimal promoter of adenovirus and a firefly luciferase reporter in the pGL3-Enhancer vector which has an SV40 enhancer. The promoterless pRL-Null plasmid containing the Renilla luciferase reporter was used as a copy number control.

Previously expression of the chimeric ULKRAB repressor in HeLa cells exhibited specific repression of the p5ULE reporter activity by 6 fold (to 16% of basal level) in a triple plasmid co-transfection of plasmids pRL-SV40 copy control, co-reporter (15 ng), pSW5UL reporter (2 µg) and pULKRAB repressor (1 µg). The ULKRAB repressor plasmid was further titrated in a similar transfection assay to optimize the level of ULKRAB expression needed for specific repression of the pSW5ULE reporter. In this experiment 2 µg pSW5ULE reporter plasmid was co-transfected with varying amounts (0 to 2 µg) of pULKRAB plasmid and 0.2 µg of co-reporter pRL-Null. The basal activities of p5ULE and p5GalE were consistent with previous observations in the absence of pULKRAB (Table ULKRAB). Specific repression mediated by ULKRAB was observed: with 0.8 µg or more of pULKRAB

pSW5UL was down regulated 20 fold (down to 5% of basal level). p5GalE was down regulated 1.7 fold (down to 62% of basal level) in the same experiment. Expression of up to 0.8 μ g of pULKRAB did not affect the expression of pRL-Null significantly in triple plasmid co-transfection (data not shown).

Table 5. Effect Of UL9-KRAB Repressor On Reporter Expression

Constructs	no pULKRAB	with pULKRAB (0.8 to about 1 μ g)
p5ULE	1 x	1/20 x (5%)
p5GalE	1 x	1/1.7 x (62%)
pRL-Null	1 x	1/1.3 x

EXAMPLE 2

Protein Displacement Studies With NF-KB

A purified Thioredoxin-p50 NF-kB fusion protein (p50C) (Genelabs Technologies, Inc.) was used to generate five oligonucleotides comprising an NF-kB DNA response element and one or two overlapping binding sites for the AT-rich binder, 21x.

The exemplified NF-kB binding sites, GGGACTTTCC (SEQ ID NO:29) and GGGATTTTCC (SEQ ID NO:30) are present in the Igk and IL-6 promoters, respectively. The exemplary oligonucleotides are presented in Fig. 7, with the transcriptional regulatory protein DNA response site indicated as bolded and uppercase, introduced compound binding sequences indicated in lowercase and potential compound binding sequences indicated as () or [].

Oligonucleotides JF101 (SEQ ID NO:31) and 102 (SEQ ID NO:32), have compound binding sequences overlapping the right side of the NF-kB DNA response element, while in the case of JF103 (SEQ ID NO:33), the overlaps are from both sides (Fig. 7).

A gel mobility shift assay was carried out as described above for UL9, and the results presented in Figs. 8A and B, indicated that: (1) 21x can efficiently displace NF-kB at concentrations as low as 1 μ M, (2) the displacement is more efficient when the NF-kB binding site is an IL-6 sequence (SEQ ID NO:30) relative to an IgK sequence (SEQ ID NO:29), and (3) 21x displaces NF-kB more efficiently than distamycin.

The native CMV promoter has 3 NFkB response sites and 1 TATA binding protein (TBP) site. Purely engineered NF-kB/TBP based 21x ligand switchable constructs were created. In each of pMC, p2MC and p4MC, 0, 2 and 4 tandem repeats of a response element consisting of the NF-kB response sequence flanked by 21x sites were fused to a CMV minimal promoter with the TBP site modified to include a 9 A/T stretch to optimize 21x binding. These promoters were cloned into pGL3-Basic to create firefly luciferase reporter constructs, as set forth below.

Firefly luciferase reporter promoter constructs containing a minimal CMV system were constructed as follows:

pMC3 (SEQ ID NO:40), which includes a minimal CMV promoter with an introduced 21x site and a luciferase reporter; p2MC5 (SEQ ID NO:41), which includes a minimal CMV promoter with an introduced 21x site and a luciferase reporter and two NFkB

sites; p4MC1 (SEQ ID NO:42), which includes a minimal CMV promoter with an introduced 21x site and a luciferase reporter plus four NFkB sites; pBKMC1 (SEQ ID NO:43), a wild type control vector which includes a minimal CMV promoter and a luciferase reporter and has a sequence of 8 to 9 A/T's near the TBP site; pBK2MC5 (SEQ ID NO:44), a control vector which includes a minimal CMV promoter, a luciferase reporter plus two tandem repeats of the NF-kB response element flanked by a poor 21x binding sequence and the flanking sequence of the TBP site was also modified to contain a 7 A/T stretch, which is less desirable for 21x binding; and pBK2MC12 (SEQ ID NO:45), a control vector which includes a minimal CMV promoter plus a luciferase reporter and two tandem repeats of the NF-kB response element.

Firefly luciferase reporter promoter constructs containing a complex CMV system were constructed as follows:

SWCMV (SEQ ID NO:46), which includes a native full CMV promoter with all 3 NFkB sites modified to contain introduced preferred binding sites for 21x and a luciferase reporter; MTCMV (SEQ ID NO:47), which includes a native full CMV promoter with all 3 NFkB sites and the TBP site modified to contain introduced preferred binding sites for 21x and a luciferase reporter; and BKCMV (SEQ ID NO:48), which includes a native full CMV promoter with 3 unmodified NFkB sites and an unmodified TBP site and a luciferase reporter.

The sequences of exemplary promoter constructs are provided below:

pSWCMV (SEQ ID NO:46), as cloned in pGL3-Basic with KpnI and HindIII sites indicated as lowercase,

TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATTAAT
ATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATT
GGCTCATGTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATA
GTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACAT
AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCATTGAC
GTCAATAATGACGTATGTTCCCATAGTAACGCAAATAGGGATTTTCCATTAACGTC
AATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCAT
ATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT
ATGCCCAGTACATGACTTTATGGGATTTTCTATTGTCAGTACATCTACGTATTA
GTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATA
GCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCATTGACGTCAATGGGAGTT
TGTTTTGGCACCAAGGTAAAAGGGATTTTCCAAAATGTCGTAACAACGCGATCG
CCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTTTATATAA
GCAGAGCTCGTTTAGTGAACCGTCAGATC

MTCMV (SEQ ID NO:47), as cloned in pGL3-Basic with KpnI and HindIII sites indicated as lowercase,

TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATTAAT
ATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATT
GGCTCATGTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATA
GTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACAT
AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCATTGAC

GTCAATAATGACGTATGTTCCCATAGTAACGCAAATATTCCCGGGAAATTAACGT
 CAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATC
 ATATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCA
 TTATGCCCAGTACATGACTTTATTCTCGAGGAATATTTGGCAGTACATCTACGTAT
 5 TAGTCATCGCTATTACCATGGTGTATGCGGTTTTGGCAGTACACCAATGGGCGTGGA
 TAGCGGTTTGACTCACGGGGATTTCGAAGTCTCCACCCCAATTGACGTCAATGGGA
 GTTTGTTTTGGCACCAAGGTAAAATTACGCGTAAAAAATGTCGTAACAACCTGCGA
 TCGCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTTGCTA
 GCCGCAGAGCTCGTTTAGTGAACCGTCAGATC

BKCMV, (SEQ ID NO:48), as cloned in pGL3-Basic with KpnI and HindIII sites indicated as lowercase,

TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAAT
 15 ATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATT
 GGCTCATGTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATA
 GTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGCGTTACAT
 AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCGCCCAATTGAC
 GTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGACGTC
 20 AATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCAT
 ATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT
 ATGCCCAGTACATGACCTTACGGGACTTTCCTACTTGGCAGTACATCTACGTATTA
 GTCATCGCTATTACCATGGTGTATGCGGTTTTGGCAGTACACCAATGGGCGTGATA
 GCGGTTTGACTCACGGGGATTTCGAAGTCTCCACCCCAATTGACGTCAATGGGAGTT
 25 TGTTTTGGCACCAAAATCAACGGGACTTTCAAAATGTCGTAACAACCTGCGATCG
 CCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAA
 GCAGAGCTCGTTTAGTGAACCGTCAGATC

Expression of the firefly reporter using various engineered minimal CMV promoter
 30 constructs was analyzed in the presence or absence of various amount of exogenous NF- κ B
 plasmid (pS50 and pS65 for the p50 and p65 NF- κ B subunit, respectively). As shown in
 Table 6, the presence of NF- κ B response elements in p2MC, p4MC, pBK2MC augmented
 the activity of the promoters approximately 4 to 17 fold relative to the activity of promoters
 lacking the NF- κ B response element (pMC and pBKMC). This effect was incrementally
 35 increased based on the number of NF- κ B response elements. These results suggest that NF- κ B
 acted as the major activator for the promoters with NF- κ B response element. Results are
 reported as normalized firefly luciferase activity relative to Renilla luciferase activity and as
 absolute firefly luciferase activity ().

Table 6 Reporter Expression Regulated By NF- κ B In A Minimal CMV System.

Construct	endogenous NF κ B	plus additional exogenous NF κ B 0.1 μ g each of pS50 and pS65)
pMC3	1 x (1 x)	2.2 x (1.3 x)
p2MC5	6 x (12 x)	44 x (32 x)
p4MC1	17 x (22 x)	85 x (65 x)
pBKMC1	1 x (1.4 x)	1.4 x (0.7 x)
pBK2MC5	3.5 x (3.8 x)	12 x (5 x)
pBK2MC12	4 x (4.4 x)	18 x (9 x)

As shown in Tables 6 and 7, the effect of additional exogenous NF- κ B p50 and p65, expression following co-transfection, further increased the activity of all the promoter constructs which have NF- κ B elements by approximately 4 to 7 fold. These results indicate that the endogenous intracellular NF- κ B level is sub-optimal for the full activation of these engineered promoters. Additional expression of exogenous NF- κ B did not significantly affect promoters without NF- κ B element.

Table 7 Reporter Expression Regulated By NF-KB In A Complex CMV System.

Construct	plus endogenous NF-kB
pBKCMV	1 x
pSWCMV	1.2~1.6 x
pMTCMV	0.4~0.5 x

Firefly luciferase reporter expression results normalized relative to co-reporter Renilla luciferase to accommodate the differential transfection efficiency in each transfection. We have analyzed the effect of expression of exogenous NF- κ B on Renilla luciferase co-reporter of pRL-Null. It was observed that with increasing amounts of NF- κ B plasmid in all co-transfections, the level of Renilla luciferase expression was decreased 3 to 7 fold. The ideal copy and transfection control co-reporter is the one that is not affected either by the transcription factors or by the ligands. However, independent of the effect of NF- κ B expression on the level of pRL-Null expression, absolute (un-normalized) expression of the firefly reporter showed a similar trend to normalized expression: that is addition of NF- κ B response elements augmented the promoter activities of the reporter constructs and additional expression of exogenously provided NF- κ B p50 and p65 increased the activity of the promoter in reporter constructs which had NF- κ B response elements, indicating the endogenous level of NF- κ B in HeLa cells is limiting for the full expression of the reporter constructs with NF- κ B response element.

EXAMPLE 3

Protein Displacement Studies With LacR

The feasibility of using LacR as an exogenous factor for a switch-on molecular switch system was evaluated using LacR, which is a repressor that represses transcription of the lac operon by binding to lacO operator sequences. Binding and displacement of LacR was tested using oligonucleotides with introduced drug binding sites that overlap the transcriptional regulatory protein binding site (Fig. 9).

In Figure 9, the transcriptional regulatory protein DNA response site is indicated as bolded and uppercase, introduced drug binding sites are indicated in lowercase and potential drug binding sites are indicated as () or []. Both of oligonucleotides tested, SEQ ID NO:34 and SEQ ID NO:35, have introduced drug binding sites which overlap the LacR binding site on both sides of the lacO sequence.

A gel mobility shift assay was carried out as described above for UL9, and the results are presented in Figs. 10A and B.

The results of the assay indicate that: (1) 21x can efficiently displace LacR, and that (2) 21x appears to displace LacR more efficiently than IPTG.

Preliminary experiments were carried out using reporter constructs. PBKLac has 3 wild type lacO response elements in an intron region of the RSV-LTR promoter fused to the firefly luciferase reporter gene. PSWLac has 3 modified lacO/21x response elements in place of wild type lacO sites. Basal activities of two clones each of pBKLac and pSWLac were determined. Two clones of pBKLac showed somewhat different activity. When compared to the expression of pBKLac34 (100%) pBKLac25 expression was 150%. Two pSWLac clones 27 and 30 each exhibited 71% and 83%, respectively. Two to four fold repression by exogenously supplied LacI was observed with as low as 0.1 μ g of pLacI together with 2 μ g of reporter construct.

EXAMPLE 4

Regulated Gene Expression In Prokaryotic Cells

The *E. coli* promoter *rrnB* P1 (SEQ ID NO:12), was selected as a prokaryotic model promoter for evaluating 21X in a cell-based aspect of the molecular switch system. The wild type UP element contains a 17 base pair stretch of AT-rich sequences, was used to test the effect of a DNA binding compound 21x, which preferably bind to AT-rich sequences (Fig 2B, SEQ ID NO:13).

The effect of 21x on the interaction of the α subunit of RNAP with the *rrnB* P1 UP element was determined by evaluating the transcriptional activity of the promoter in several *E. coli* strains carrying a wild type or mutant *rrnB* P1 promoter fused to a *lacZ* reporter on its chromosome, as a phage monolysogen.

The promoters which were evaluated include a wild type *rrnB* P1 promoter (RLG3074, SEQ ID NO:15), which has a consensus UP sequence at a distal site, two mutant *rrnB* P1 promoters which have a consensus UP sequence at both proximal and distal sites (RLG4192, SEQ ID NO:16 and RLG4174, SEQ ID NO:17), and the "core" *rrnB* P1 promoter (RLG3097, SEQ ID NO:14), which functions as a negative control and lacks an UP sequence and a 21X binding site [Table 8 and Fig. 4A, wherein 21x binding sites are indicated as ()].

Table 8

UP region sequence		Relative Basal Activity	
RLG3097	GACTGCAGTGGTACCTAGGAGG	(SEQ ID NO:14)	1 X
RLG3074	AG(AAAATTATTTTAAATTT)CCT	(SEQ ID NO:15)	30 X
RLG4192	GG(AAAATTTTTCATTTTCAAAA)GTA	(SEQ ID NO:16)	110 X
RLG4174	TG(AAATTTATTTT)GCGAAAGGG	(SEQ ID NO:17)	75 X

Figure 4B shows the results of testing the activity of *E. coli* strains that carry the various *rrnB* P1 promoters fused to a *lacZ* reporter with 21X.

The promoter activity of RLG3097 (SEQ ID NO:14), which has the "core" sequence was not affected by 21x.

E. coli strains that carry *rrnB* P1 promoters which have a distal UP element (RLG4174, SEQ ID NO:17) or both proximal and distal UP elements (RLG 3074, SEQ ID NO:15 and RLG4192, SEQ ID NO:16), exhibited similarly significant down-regulation of reporter gene expression, when treated with 21x.

5 The results indicate that targeting RNA polymerase α sites in the *E. coli* *rrnB* P1 promoter with a small DNA-binding molecule, exemplified by 21x, may be used to effectively regulate prokaryotic gene expression in the chromosomal context.

Such targeting studies also suggest that a strong promoter like *rrnB* P1, and engineered variants thereof, can be down-regulated with a sequence preferential DNA-binding small molecule when the engineered promoter contains a small molecule binding sequence near the protein binding site.

EXAMPLE 5

Regulated Gene Expression Using The Cyclin D1 Promoter

15 A full-length 1900-bp fragment of the human cyclin D1 promoter representing nucleotides -1745 to +155 relative to the transcription start site and a series of cyclin D1 5' promoter deletions were constructed and PCR amplified. The -1745 wild-type and various site-directed mutants of the cyclin D1 promoter were inserted into the promoter-less firefly luciferase plasmid (pGL3-basic) and co-transfected into MCF7 cells human breast carcinoma cells, which overexpress cyclin D1, together with an SV40 promoter driven Renilla luciferase control plasmid. Firefly luciferase activity for each construct was normalized to Renilla luciferase activity and compared to that of the full-length wild-type promoter (-1745). The data are presented as the mean \pm SEM for a minimum of two independent transfections done in triplicate. The promoter constructs were assayed in MCF7 cells, a second cyclin D1 overexpressing breast carcinoma cell line, ZR75; a breast cell line that expresses cyclin D1 normally, HMEC; a cyclin D1 overexpressing colon cancer cell line, HCT116; and a cyclin D1 overexpressing pancreatic cancer cell line, PANC-1.

20 The human breast carcinoma cell lines MCF7 and ZR75 were maintained in DMEM/F12 medium with 10% fetal bovine serum, 10 μ g/ml bovine insulin and antibiotics (penicillin/ streptomycin). The human colon carcinoma cell line HCT116 was maintained in McCoy's medium with 10% fetal bovine serum and pen/strep. The human pancreatic cell line PANC-1 was maintained in DMEM/F12 with 10% fetal bovine serum and pen/strep. Human mammary epithelial cells (HMEC) were maintained in Epithelial Growth Media supplemented with bovine pituitary extract (50 μ g/ml), hydrocortisone (500ng/ml), hEGF (10ng/ml), and insulin (5 μ g/ml). All lines were maintained at 37°C, 5% CO₂. MCF7, ZR75, HCT116 and PANC-1 cells were purchased from the American Type Culture Collection. HMEC cells were purchased from Clonetics Corp.

35 Cells were transiently transfected with LipofectAMINE (GIBCO Life Sciences) in triplicate in 6-well tissue culture plates (Corning, NY). Equal numbers of cells (3×10^5 /well) were seeded in each well, 24 hours prior to transfection. Prior to transfection, cells were equilibrated in 800 μ l fresh medium (OptiMEM with 5% FBS and pen/strep). Cells were transfected with 5 μ g of reporter plasmid containing a cyclin D1 promoter constructs in 200 μ l transfection buffer. After 4 hours incubation with the transfection solution, cells were fed with

4 ml OptiMEM with 5% FBS and pen/strep. Cells were harvested 48 hours after transfection.

Following co-transfection into various cell lines, the cyclin D1 promoter constructs containing a mutation of the CRE and/or a mutation of the -30 to -21 region resulted in a reduction in luciferase activity, suggesting that both the CRE and the -30 to -21 sites are involved in transcriptional regulation of cyclin D1 basal expression in all of the overexpressing cancer cell lines tested, as well as in HMEC cells which express normal levels of cyclin D1.

Site-directed mutagenesis of the -30 to -21 promoter region was carried out and constructs assayed in MCF7 cells. The assay results indicate that bases between -30 and -24 (GAGTTT) are the most important for transcriptional activation from this site (Table 9).

Table 9 Reporter Activity Of Cyclin D1 Promoter Constructs

Construct	Mutations in -30-21 region	% Wild Type Activity
WT -1745	GAGTTTTGTT	100
-30 -21 -1745	TCTGGGATCC	33 +/- 2.2
-30 -26 -1745	TCTGGTTGTT	43 +/- 3.5
-25 -21 -1745	GAGTTGGCGG	34 +/- 4.7
-30 -28 -1745	TCTTTTTGTT	33 +/- 6.3
-28 -23 -1745	GATGGGATTT	46 +/- 5.1
-23 -21 -1745	GAGTTTTTCC	138 +/- 16.4
10 bp 21x -1745	GAGTTTTTTTAAAG	87 +/- 11.4
8 bp 21x -1745	GAGTTTTAAAAGAG	85 +/- 7.8

A dimer of netropsin, designated 21x, which has a high affinity for A/T-rich DNA sequences and has been shown to footprint a DNA site of about 10bp was used to regulate cyclin D1 promoter activity. A detailed biochemical characterization of 21x is provided in co-owned USSN 06/154,415, expressly incorporated by reference herein.

Oligonucleotide binding sites for the netropsin dimer 21x, were introduced overlapping the -30 to -21 region of the CCND1 promoter. In one case, the site was introduced into the 3' end of the A/T-rich -30 to -21 site, by changing only 2bp (10 bp 21x, SEQ ID NO:37). A second 21x binding site was constructed by mutating 5 bp of the wild-type promoter sequence to produce an uninterrupted 8 A/T stretch (8 bp 21x, SEQ ID NO:38). Binding of 21x to these sites was confirmed using a hybridization stabilization assay, as detailed herein and described in co-owned application USSN 09/151,890 and USSN 09/393,783, incorporated herein by reference. Both 21x site-containing constructs were cloned in the context of the -1745 cyclin D1 promoter in pGL3 basic, transfected into MCF7 cells and demonstrated to retain high levels of promoter activity in MCF7 cells in the absence of 21x (85% and 87% of wild-type promoter activity respectively).

When transiently transfected MCF7 cells were treated with 0, 1 or 10 μ M 21x and assayed after 48 hr, activity of the wild-type cyclin D1 promoter constructs was unaffected by 21x, activity of the -30 to -21 mutant construct was approximately 25% of wild type and unaffected by 21x treatment, while both the 8 bp 21x (SEQ ID NO:38) and 10 bp 21x (SEQ ID NO:37) constructs showed reduced promoter activity at 1 μ M 21x and levels as low as those of

the -30 to -21 mutant construct at 10 μ M 21x (Fig. 12).

The results of luciferase expression assays in mammalian MCF7 cells indicate that 21x treatment is effective to specifically lower cyclin D1 promoter activity 4-fold when a 21x-binding site is present overlapping the -30 to -21 transcriptional activator DNA response site, while promoter constructs lacking the 21x sites were unaffected (Fig. 12)..

The results show that it is possible to specifically down-regulate overexpressed endogenous cyclin D1 in tumor cells by developing a DNA-binding compound with specificity for a regulatory sequence of the promoter.

EXAMPLE 6

Regulated Gene Expression Using the HBV core Promoter

A luciferase reporter construct was constructed with a linearized full-length copy of the HBV genome, with the core promoter positioned immediately upstream and driving the expression of the reporter. Mutagenic primers containing blocks of 15 nucleotides of targeted sequence mutation were designed to generate a series of linker scanner mutant promoter reporter clones using either a *Morph*TM (5'Prime to 3'Prime, Boulder, CO) or a QuikChangeTM (Stratagene, La Jolla, CA) mutagenesis protocol.

Targeted segments of the promoter found to be resistant to mutagenesis were further sub-divided into smaller blocks of mutations consisting of 7-8 nucleotides. This series of linker scanner clones span the entire length of the core promoter segment. Mutagenic primers were also used to construct site-directed mutant constructs of known transcription factor binding sites including the hepatocyte nuclear factor sites, HNF3 and HNF4.

To determine potential critical regulatory elements in the core promoter, linker scanner analysis was performed using the series of systemic mutation clones constructed. Each linker scanner mutant construct was evaluated for promoter activity in transient transfection experiments based on luciferase reporter activity in the hepatoma-derived cell lines HepG2 and HuH7. The HBV stably-transfected cell lines, 22.1.5 and HepAD38, were also used in the linker scanner analysis. An increase or decrease in relative luciferase reporter activity relative to the wild type indicates potential presence of control elements critical to regulation of gene transcription.

Three regions of interest were identified by linker scanning analysis. All 3 regions align with cis-elements previously reported in the literature. One region contains sequences corresponding to a HNF4 transcription factor binding site (SEQ ID NO:50). A second region contains sequences corresponding to a proximal HNF3 transcription factor binding site (SEQ ID NO:48). Both of these protein factor sites have been described as important activation elements for the HBV core promoter. Mutation of a third region abolished the wild type TATA box sequence (SEQ ID NO:51) of the promoter. A second HNF3 site (Distal HNF3-1) has been reported, however, mutation of the distal HNF3 site did not show any adverse effects in promoter activity (Table 10).

Table 10. Reporter Analysis of Site-Directed Mutants of HNF3 and HNF4 Sites of the HBV Core Promoter.

	Nucleotide Coordinates (HBV ayw Strain)	Site-Directed Mutant Sequence	Percent Wild Type HepAD38
Distal HNF3	1680 – 1691	CCAGGGCCCCGA	102
Proximal HNF3	1715 – 1726	GCCGCGGTCTGT	33
HNF4	1661 – 1672	CGTCCGCGGTGA	29

Following identification of the TATA box and the HNF4 and proximal HNF3 sites as the control elements most critical for core promoter activity, transcriptional activation as a result of the binding of the TATA binding protein (TBP) and the HNF transcription factors was further studied. It will be appreciated that failure of these protein factors to bind would result in down-regulation of the promoter.

Small DNA-binding compounds were utilized to test their ability to alter the transcription level from wild type and engineered HBV core promoters, either by interference and/or displacement of protein factor binding to its cognate nucleotide binding sequences. The nucleotide composition at the core TATA box contains a run of seven (7) A and T bases that could serve as a binding site for the compound 21x, which exhibits a binding preference of A/T-rich sequences. As shown in Table 11, 21x down-regulated the core wild type promoter by approximately 50% in transient transfection assays at concentrations of 0.5-1 μ M. An engineered promoter construct, TATA21xR (SEQ ID NO:52) was prepared containing an introduced 21x binding site located adjacent to and overlapping the TATA box sequence. The down-regulating effects were pronounced for cells transfected with the engineered TATA21xR construct, for which the reporter gene activity decreased by 4-5 fold, consistent with the premise that 21x may bind with higher affinity to the A/T-rich binding sequence present in TATA21xR than to the core TATA box native sequence, leading to enhanced interference and/or displacement of TBP binding to the DNA.

A promoter construct, TATAmut (SEQ ID NO:53), with the TATA box sequence mutated in a manner to abolish TBP binding exhibited a low level of transcription and was not responsive to 21x treatment. Another mutant construct, 3'TATAmut (SEQ ID NO:54), with a sequence alteration resulting in a shorter run of A/T nucleotides downstream of the TATA box also showed no effects upon 21x treatment. The DNA-binding compound (21x) is shown to be capable of altering levels of gene transcription through its interaction with a basal transcription factor.

Table 11. 21x Down-regulates Expression of the HBV Core Promoter Through the TATA Box

Reporter Construct	Sequence	Percent Wild Type Promoter Activity	
		No Treatment	Treatment with 1 μ M 21x
Wild type ²	TACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATG	100	60
TATA _{mut} ³	TACTAGGATTAGTGCTTAAGCCCTTGGTCTGCGCACCA GCACCATG	15	13
3'TATA _{mut} ⁴	TACTAGGAGGCTGTAGGCATAAAGCTCGAGTATACAAC GCACCATG	31	36
TATA _{21xR} ⁵	TACTAGGAGGCTGTAGGCATAAATTAGTCTGCGCACCAGCACCATG	98	21

Another DNA-binding compound, GL046732, was demonstrated to be effective in the regulation of promoter activity of HBV core promoter constructs with engineered compound binding sequences. Three types of potential compound binding sequences were designed and position-cloned to be adjacent and overlapping transcription factor recognition sites. The general designs of the three different types of potential compound binding sequences are (ds1) two core sequences of 5 A/T nucleotides on either end with a center block of 3 G/C nucleotides, (ds2) a run of 12 to 13 A/T nucleotides, and (ds3) a run of 8 to 9 A/T nucleotides. Exemplary promoter constructs include the following:

TATARds1 (SEQ ID NO:55)

TACTAGGAGGCTGTAGGCATAAATGCGTAAAAGCACCAGCACCATGCAAC

TATARds2 (SEQ ID NO:56)

TACTAGGAGGCTGTAGGCATAAATTAATAACGCACCAGCACCATGCAAC

TATARds3 (SEQ ID NO:57)

TACTAGGAGGCTGTAGGCATAAATTAATCCGCGCACCAGCACCATGCAAC

As shown in Table 12 and Figure 13, the DNA-binding compound GL046732 used to treat HepG2 cells transfected with wild type and engineered core promoter constructs, preferentially down-regulated the promoter activity of the TATARds1 clone (SEQ ID NO:55) in a dosage-dependent manner resulting in a 4 fold reduction in promoter activity at the 40 μ M concentration. The promoter activity of clone TATARds3 (SEQ ID NO:57) was also affected, but the level of down-regulation observed was less of that seen for the "ds1"

² Wild type=wild type core promoter (SEQ ID NO:51)

³ TATA_{mut}=mutant construct with TATA (SEQ ID NO:53)

⁴ 3'TATA_{mut}=mutant construct with 15 nucleotides downstream from TATA box mutated (SEQ ID NO:54)

⁵ TATA_{21xR}=construct with engineered 21x site on right side of TATA (SEQ ID NO:52)

sequence. The core promoter activity of the wild type construct remained relatively unaffected.

Table 12. Effects of GL046732 on Promoter Activity of Core Promoter Constructs
Containing Engineered Drug-Binding Sites

Reporter Construct	Percent of no Drug Control		
	1 μ M GL046732	10 μ M GL046732	40 μ M GL046732
Wild type			
TATARds1	114	67	93
TATARds2	56	39	25
TATARds3	71	62	65
	102	73	39

Similarly, ds1, ds2, and ds3 sequences were designed and placed adjacent and overlapping the proximal HNF3 site. Exemplary engineered sequences include the following:

HNF3Rds1 (SEQ ID NO:58)

ACCTTGAGGCATACTTCAAAGACTGTTGATTTAGCGAATAAGAGGAGTTGG

HNF3Rds2 (SEQ ID NO:59)

ACCTTGAGGCATACTTCAAAGACTGTTTATTTTAATAACGGGAGGAGTTGG

HNF3Rds3 (SEQ ID NO:60)

ACCTTGAGGCATACTTCAAAGACTGTTTATTTAAGGACTGGGAGGAGTTGG

Oligonucleotides containing these HNF3 engineered sequences were used along with a wild type oligomer in an *in vitro* gel mobility shift assay, and found to bind the HNF3 transcription factor specifically. GL046732 was then tested for its ability to bind to the engineered sequences and either cause displacement of HNF3 or prevent the transcription factor from binding. GL046732 was found to be most effective in displacement of protein-bound band in the gel shift assay with the same drug sequence (ds1). The EC₅₀ value for protein displacement was determined to be in the concentration range of 300-800 nM. Similar to the transfection results obtained from the TATAds constructs, GL046732 was also slightly effective in displacement of HNF3 with the ds3 type sequence, while having no effects on the wild type sequence.

These results, taken together, indicate that a compound binding site may be engineered into a promoter and thereby serves as a means for regulated gene expression of a coding sequence operably linked to it.

SEQUENCE LISTING TABLE
(all oligonucleotides shown as single stranded in 5' to 3' direction)

Description	SEQ ID NO
UL9 DNA response element CGTTCGCACTT (11 bp)	1
GAL4 DNA response element CGGAGTACTGTCCTCCG (17 bp)	2
ZFHD1 DNA response element TAATTANGGGNG (12 bp)	3
NF-KB p65 Genbank Accession Number HUM65NFKB	4
tetO DNA response element TCCCTATCAGTGATAGAGA (19 bp)	5
lacO DNA response element CTTAACACTCG:CGAGTGTTAAG (22 bp)	6
Ecdysone receptor RG(GT)TCANTGA(CA)CY (15 bp)	7
VP16: aa 413-489 reference or sequence	8
VP64: tetramer of aa 437-447 of VP16	9
KRAB: aa 1-97 reference or sequence	10
Mad: aa 1-36 reference or sequence	11
Sequence of <i>rrnB</i> P1 promoter: from -66 to +50 CGCGGTCAGAAAATTATTTTAAATTTCTTGTTCAGGCCGGAATAACTCCCTATAATG CGCCACCACTGACACGGAACAACGGCAAACACGCCGCCGGGTGACGGGGTTCTCCT	12
<i>rrnB</i> P1 promoter UP element AGAAAATTATTTTAAATTTCTT	13
RLG3097 (core) GACTGCAGTGGTACCTAGGAGG	14
RLG3074 (WILD TYPE) AG(AAAATTATTTTAAATTT)CCT	15
RLG4192 GG(AAAATTTTTTTTCAAAA)GTA	16
RLG4174 TG(AAATTTATTTT)GCGAAAGGG	17
modified UL-9 DNA response sequence TGTTCGCACTT	18
modified UL-9 DNA response sequence (YK 202LX, 52-mer) CATGGACG CCACTG AGCCGTTT TGTTCGCACTT GAGGCGAGTCGATGCACC	19
modified UL-9 DNA response sequence (YK 202RX-A, 54-mer) CATGGACG CCACTG AGCCG TGTTCGCACTT TTTTGGGCGAGTCGATGCACC	20
modified UL-9 DNA response sequence (YK 202RX, 58-mer) CATGGACG CCACTG AGCCGTTTT TGTTCGCACTT TTTTGGGCGAGTCGATGCACC	21
MEF C(TTAAAAATAA)C	22
780BP (TTGAAAAATCAA)CGCT	23
UL9 (modified) (tttTGTT)CGCAC(TTttttt)	24
NFkB (modified) (ttttGGG[AtTTT]CCtttt)	25
LacO (modified) (aaaaAATT)GTGAGCGCTCAC(AATTttt)	26
NtBBF1 (plant tissue-specific transcription factor) ACTTTA	27
DRE (plant element identified in the promoter region of the <i>rd29A</i> gene associated with dehydration and cold-induced gene expression) TACCGACAT	28
NF-kB DNA response sequence from Igk promoter: GGGACTTTCC	29
NF-kB DNA response sequence from IL-6 promoter: GGGATTTTCC	30
JF101 (NFKB1) (50mer) (right side) cgac cgtgctcgag TTAACGGGACTTTCCAAaaa cgatcg gact ggactc	31
JF 102 (NFKB2)(60mer)(right side) cgac cgtgctcgag TTAACGGGAiTTTCCAAaaa cgatcg gact ggactc	32
JF 103 (NFKB3)(60mer) (both sides) cgac cgtgctcgag aaatiGGGAiTTTCCAAaaa cgatcg gact ggactc	33
LacI aaaaAATTGTGAGCGCTCACAATTttt	34
LacI ttttTTGTGAGCGGATAACAAaa	35
Cyclin D1 -30-21 TCTGGGATCC	36
Cyclin D1 10bp 21x GAGTTTTTTTTTAAG	37
Cyclin D1 8bp 21x GAGTTTAAAGAG	38
NFKB p50 Genbank Accession Number HUMNFKB34	39

Description	SEQ ID NO
NFKB pMC3 (NheI to BglI) GCTAGCCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGTTTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCAGATCT	40
NFKB 2MC5 (NheI to BglI) GCTAGCGCCCAAATTGGGATTTTCCAAAAAGCCGAAATTGGGATTTTCCAAAAACCGCCGATCGCCC GCCCGTTGACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGT TTATATAAGCAGAGCTCGTTTAG TGAACCGTCAGATCAGATCT	41
NFKB 4MC1 (MluI to BglI) ACGCGTGCCCAAATTGGGATTTTCCAAAAAGCCGAAATTGGGATTTTCCAAAAACCGCGCTAGCGCC CAAATTGGGATTTTCCAAAAAGCCGAAATTGGGATTTTCCAAAAACCGCCGATCGCCCCGCCCGTTG ACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGT TTATATAAGCAGAGCTCGTTTAGTGAACCGTC AGATCAGATCT	42
NFKB BKMC1 (NheI to BglI) GCTAGCCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCAGATCT	43
NFKB BK2MC5 (NheI to BglI) GCTAGCGCCCAGGTCGGGATTTTCCGAGGAGCCGAGGTCGGGATTTTCCGAGGACCGCCGATCGCCC GCCCGTTGACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGCCATATATAAGCAGAGCTCGTTTAG TGAACCGTCAGATCAGATCT	44
BK2MC12 (NheI to BglI) GCTAGCGCCCAGGTCGGGATTTTCCGAGGAGCCGAGGTCGGGATTTTCCGAGGACCGCCGATCGCCC GCCCGTTGACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGCCATATATAAGCAGAGCTCGTTTAG TGAACCGTCAGATCAGATCT	45
NFKB SWCMV	46
NFKB MTCMV	47
NFKB BKCMV	48
HBV core proximal, HNF3-2 binding site (GACTGTTTGTTC)	49
HBV core HNF4 binding site (AGGACTCTTGGA)	50
HBV core WT TACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATG	51
HBV core TATA21xR TACTAGGAGGCTGTAGGCATAAATTAGTCTGCGCACCAGCACCATG	52
HBV core TATAmut (TACTAGGATTAGTGCTTAAGCCCTTGGTCTGCGCACCAGCACCATG)	53
HBV core 3'TATAmut (TACTAGGAGGCTGTAGGCATAAAGCTCGAGTATACAACGCACCATG)	54
HBV core TATARds1 TACTAGGAGGCTGTAGGCATAAATGCGTAAAAGCACCAGCACCATGCAAC	55
HBV core TATARds2 TACTAGGAGGCTGTAGGCATAAATTAATAAACGCACCAGCACCATGCAAC	56
HBV core TATARds3 TACTAGGAGGCTGTAGGCATAAATTAATCCGCGCACCAGCACCATGCAAC	57
HNF3Rds1 ACCTTGAGGCATACTTCAAAGACTGTTGATTTAGCGAATAAGAGGAGTTGG	58
HNF3Rds2 ACCTTGAGGCATACTTCAAAGACTGTTTATTTAATAACGGGAGGAGTTGG	59
HNF3Rds3 ACCTTGAGGCATACTTCAAAGACTGTTTATTTAAGGACTGGGAGGAGTTGG	60
pACTULVP activator construct-Figs 14A/B	61
pACT ULKRAB repressor construct-Figs 15A/B	62

IT IS CLAIMED:

1. A molecular switch, comprising:
a first nucleic acid construct having

5 (i) a DNA response element for a transcriptional regulatory protein operably linked to a first promoter;

(ii) a compound binding sequence in the vicinity of said DNA response element for binding to a DNA binding compound;

(iii) a transgene under the control of said first promoter; and

10 (iv) a DNA binding compound.

2. The molecular switch according to claim 1, further comprising:

(v) a second nucleic acid construct having the coding sequence for a transcriptional regulatory protein operably linked to a second promoter.

15

3. The molecular switch according to claim 1 or 2, wherein said transcriptional regulatory protein is a native protein.

4. The molecular switch according to claim 2, wherein said transcriptional regulatory protein is a synthetic or engineered protein.

20

5. The molecular switch according to claim 2, wherein said second promoter is a constitutive promoter.

25

6. The molecular switch according to claim 2, wherein said second promoter is a regulatable promoter.

7. The molecular switch according to claim 2, wherein a single vector includes said first and second nucleic acid constructs.

30

8. The molecular switch according to claim 2, having a first vector including said first nucleic acid construct and a second vector including said second nucleic acid construct.

9. The molecular switch according to claim 1, wherein said first nucleic acid construct is an adenovirus vector.

35

10. The molecular switch according to claim 1, wherein said first nucleic acid construct is an adeno-associated virus vector.

11. A molecular switch, comprising:
a first nucleic acid construct having

40

(i) a DNA response element for a transcriptional regulatory protein operably linked to a regulatable promoter;

- (ii) a compound binding sequence in the vicinity of said transcriptional regulatory protein DNA response element for binding to a DNA binding compound;
(iii) a transgene and the coding sequence for a transcriptional regulatory protein under the control of said regulatable promoter; and
5 (iv) a DNA binding compound.

12. The molecular switch according to claim 11, further comprising:

- (v) the coding sequence for a transcriptional regulatory protein operably linked to said regulatable promoter.

10 13. The molecular switch according to claim 1 or 11, wherein said nucleic acid construct has from 1 to 12 compound binding sequences.

15 14. The molecular switch according to claim 1 or 11, wherein compound binding sequence has from about 8 to 20 nucleotides.

15 15. The molecular switch according to claim 1 or 11, wherein said nucleic acid construct has from 1 to 12 tandem repeated transcriptional regulatory protein DNA response elements.

20 16. A cell comprising the molecular switch according to claim 1 or 11.

25 17. A cell according to claim 16, wherein said cell is selected from the group consisting of a plant cell, an animal cell, a yeast cell, a bacterial cell, an insect cell and an archaea cell.

18. A method of producing a cell having a molecular switch for modulating gene expression, said method comprising:

30 (i) transforming said cell with a nucleic acid construct having a DNA response element which binds a transcriptional regulatory protein operably linked to a promoter, a compound-binding sequence in the vicinity of said DNA response element for binding to a DNA binding compound, a transgene under the control of a promoter; and

35 (ii) exposing said transformed cell to a DNA binding compound, wherein binding of the DNA binding compound to said compound binding sequence is effective to inhibit binding of a transcriptional regulatory protein to the DNA response element, thereby derepressing or deactivating expression of the gene, where the transcriptional regulatory protein is a repressor or activator protein, respectively.

19. The method according to claim 18, comprising:

40 (v) further transforming said cell with a second nucleic acid construct having a nucleic acid sequence encoding a transcriptional regulatory protein operably linked to a second promoter.

20. A method of modulating expression of an exogenous gene in a cell by a DNA binding-compound, comprising:

adding said DNA binding-compound to a cell which expresses a transcriptional regulatory protein, and is transformed with a genetic construct having a DNA response element which binds said transcriptional regulatory protein operably linked to a promoter, a compound-binding sequence in the vicinity of said DNA response element for binding to said compound, and a transgene under the control of said promoter,

wherein addition of said DNA binding compound to said cell, and binding of the compound to said compound-binding sequence is effective to inhibit binding of said transcriptional regulatory protein to the DNA response element, thereby derepressing or deactivating expression of the exogenous gene, where the transcriptional regulatory protein is a repressor or activator protein, respectively.

21. The molecular switch according to claim 1 or 11, wherein said transcriptional regulatory protein has a DNA binding sequence selected from the group consisting of a UL9 sequence, an NF- κ B sequence, a GAL4 sequence, a ZFHD1 sequence, a LacR sequence, a TetR sequence, a LexA sequence, and the ecdysone receptor binding sequence.

22. The cell according to claim 16, wherein the DNA binding sequence of said transcriptional regulatory protein is selected from the group consisting of a UL9 sequence, an NF- κ B sequence, a GAL4 sequence, a ZFHD1 sequence, a LacR sequence, a TetR sequence, a LexA sequence, and the ecdysone receptor binding sequence.

23. The molecular switch according to claim 1 or 11, wherein said regulatory domain is an activator domain selected from the group consisting of VP16, NF-KB, Gal4, TFE3, ITF1, Oct-1, Sp1, Oct-2, NFY-A, ITF2, c-myc, and CTF.

24. The cell according to claim 16, wherein the regulatory sequence of said of said transcriptional regulatory protein is an activator selected from the group consisting of VP16, NF-KB, Gal4, TFE3, ITF1, Oct-1, Sp1, Oct-2, NFY-A, ITF2, c-myc, and CTF.

25. The molecular switch according to claim 1 or 11 wherein the regulatory sequence of said of said transcriptional regulatory protein is a repressor selected from the group consisting of Kruppel (KRAB), kox-1, TetR, even-skipped, LacR, engrailed, hairy (HES), Groucho (TLE), RING1, SSB16, SSB24, Tup1, Nab1, AREB, E4BP4, HoxA7, EBNA3, Mad and v-erbA.

26. The cell according to claim 16, wherein the regulatory sequence of said of said transcriptional regulatory protein is a repressor selected from the group consisting of Kruppel (KRAB), kox-1, TetR, even-skipped, LacR, engrailed, hairy (hes), Groucho(TLE), RING1, SSB16, SSB24, Tup1, Nab1, AREB, E4BP4, HoxA7, EBNA3, Mad and v-erbA.

27. The molecular switch according to claim 1 or 11, wherein said DNA response

element is characterized by a series of from 1 to 12 repeated transcriptional regulatory protein binding sites.

5 28. The cell according to claim 16, wherein said DNA response element is characterized by a series of from 1 to 12 repeated transcriptional regulatory protein binding sites.

29. The molecular switch according to claim 1 or 11, wherein said compound-binding sequence is from about 8 to 20 nucleotides.

10

30. The cell according to claim 16, wherein said compound-binding sequence is from about 8 to 20 nucleotides.

15

31. A method of screening DNA binding compounds for the ability to regulate a molecular switch, comprising:

(i) identifying a DNA sequence to which a DNA binding compound is to bind;

(ii) providing a nucleic acid construct having a DNA response element for a transcriptional regulatory protein and a compound binding sequence in the vicinity of said DNA response element; and

20

(iii) screening a plurality of candidate DNA binding compounds, by exposing each of the candidate compounds to said nucleic acid construct and identifying DNA binding compounds having the ability to bind to the compound-binding sequence.

25

32. The method according to claim 31, further comprising:

(iv) combining a transcriptional regulatory protein with said nucleic acid construct, and identifying and selecting DNA binding compounds having the ability to bind to displace said transcriptional regulatory protein from said DNA response element.

30

33. The method according to claim 32, further comprising:

35

(v) modifying said nucleic acid construct to further include a transgene under the control of a promoter, wherein said transgene is a reporter gene, and identifying and selecting DNA binding compounds having the ability to inhibit binding of said transcriptional regulatory protein to said DNA response element, as evidenced by derepression or deactivation of expression of the reporter gene, where the regulatory protein is a repressor or activator protein, respectively.

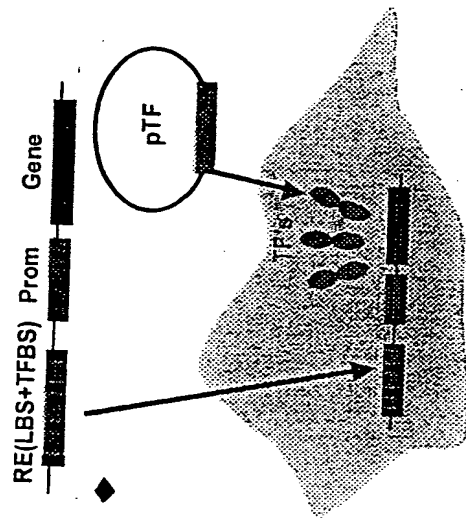


Figure 1

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distal UP
nnAAA(A'D)(A'D)U(A'D)TTTTnnAAAAnnn
 proximal UP

FIGURE 2A

-66 -59 UP element -38
 CCGGCAAGAAATATTAAATTCCTTCTGACGCGCAATACGCCCA
 AATGGGAC
 +1
 CACGACACCGAACACCGGAAACACCGCGCGGACGCGGTTCTCT
 +50

FIGURE 2B

Switchable Promoter: Drug Targeting near *Cis* Element

•Direct targeting

MEF	<u>C(TTAAAAAATAA)C</u>
780BP	<u>(TTGAAAAAATCAA)CGCT</u>

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•Overlapping Targeting (test for up or down-stream)

UL9	(<u>ttttTGTT</u>) <u>CGCAC(TT</u> ttttt)
NF _k B	(<u>ttttGGG</u> [<u>ATTT</u>]) <u>CC</u> ttttt]
LacO	(<u>aaaaAATT</u>) <u>GTGAGCGCTCAC</u> (<u>AATT</u> tttt)
NtBBF1	(<u>tttACT</u> [<u>TTA</u>])tttt]

Figure 3

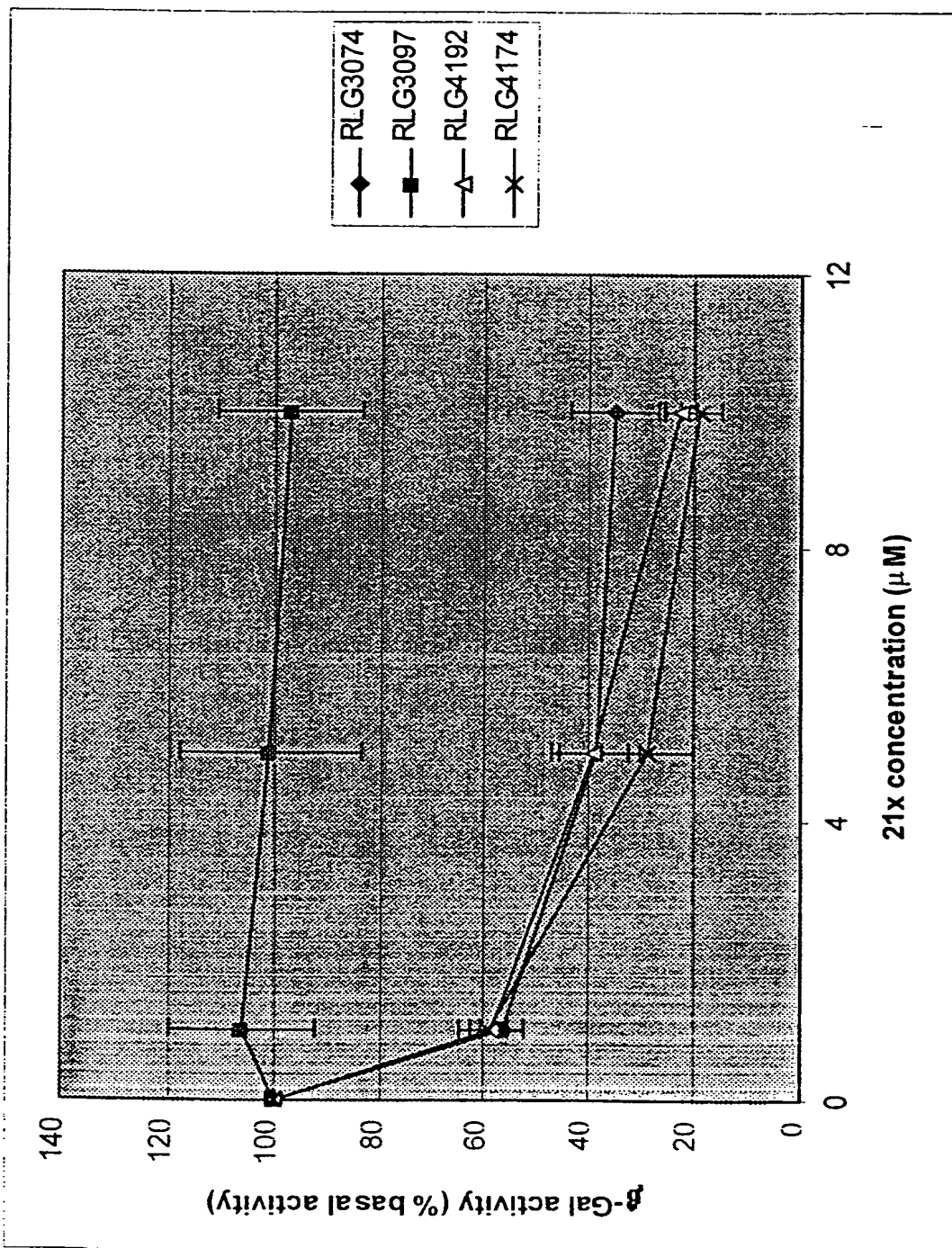
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rrnB P1 promoter UP Sequences

RLG3097 (core)	GACTGCAGTGGTACCTAGGAGG
RLG3074 (wt)	AGAAATTTATTTTAAATTTTCCT
RLG4192	GGAAATTTTTTTCAAAAGTA
RLG4174	TGAAATTTATTTTTCGAAAGGG

Figure 4A

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**Figure 4B**

YK 202LX (52-mer) 5' CATGGACG CCACTG AGCCGTTTT **TGTTCGCACTT** GAGGCGAGTCGATGCACC 3'
3' GTACCTGC GGTGAC TCGGCAAAA **ACAAGCGTGAA** CTCCGCTCAGCTACGTGG 5'

YK 202RX-A (54-mer) 5' CATGGACG CCACTG AGCCG **TGTTCGCACTT** TTTTGTGAGCGGAGTCGATGCACC 3'
YK 202RX-B (54-mer) 3' GTACCTGC GGTGAC TCGGC **ACAAGCGTGAA** AAAAACTCCGCTCAGCTACGTGG **B** 5'

YK 202LRX (58-mer) 5' CATGGACG CCACTG AGCCGTTTT **TGTTCGCACTT** TTTTGTGAGCGGAGTCGATGCACC 3'
3' GTACCTGC GGTGAC TCGGCAAAA **ACAAGCGTGAA** AAAAACTCCGCTCAGCTACGTGG 5'

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Figure 5

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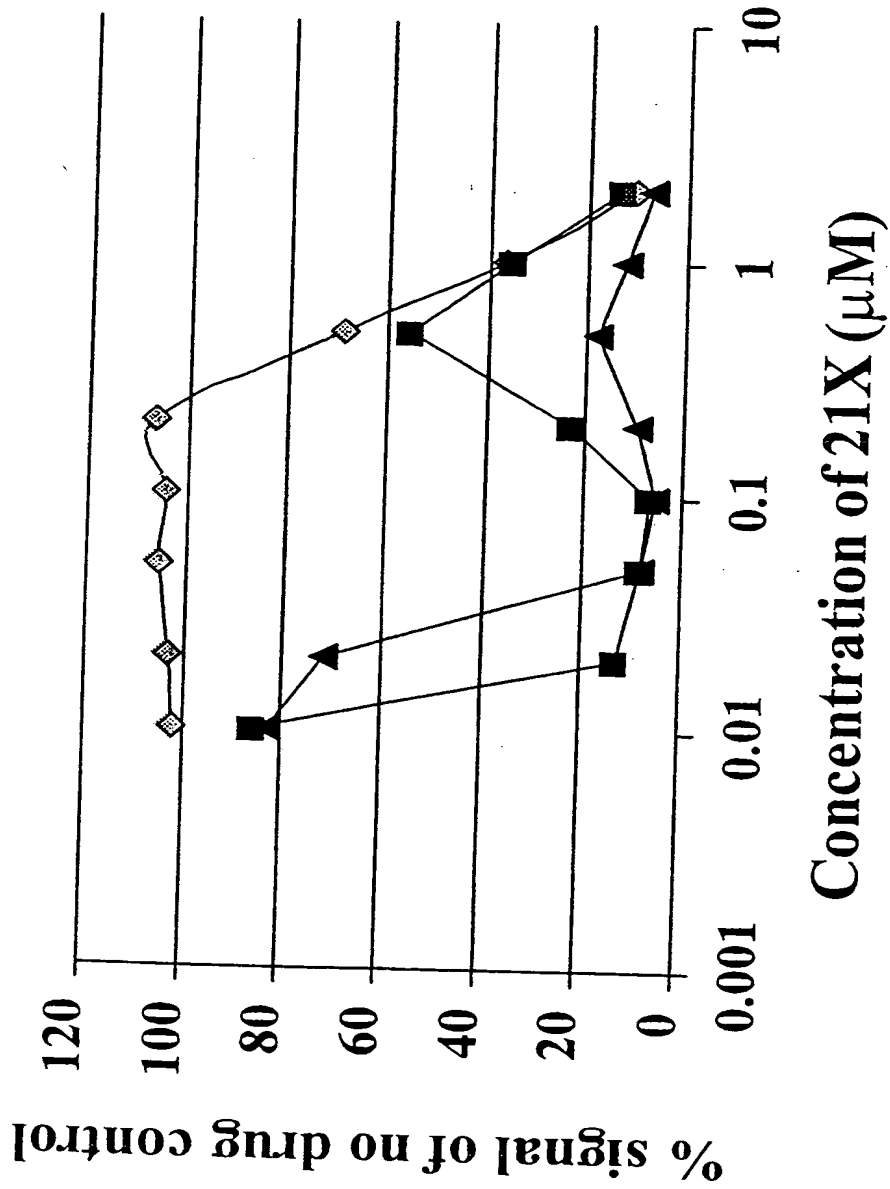


FIGURE 6

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JF 101 (NFKB1) (50mer) (right side)

5' cgac cgtgctcgag **TTAACGGGACTTCCAAaaa** cgatcg gact ggactc 3'
3' gctg gcacgagctc **AATTGCCCTGAAGGTTttt** gctagc ctga cctgag 5'

JF 102 (NFKB2) (60mer) (right side)

5' cgac cgtgctcgag **TTAACGGGACTTCCAAaaa** cgatcg gact ggactc 3'
3' gctg gcacgagctc **AATTGCCCTaAAAGGTTttt** gctagc ctga cctgag 5'

JF 103 (NFKB3) (60mer) (both side)

5' cgac cgtgctcgag **aaattGGGACTTCCAAaaa** cgatcg gact ggactc 3'
3' gctg gcacgagctc **tttaacCCCTaAAAGGTTttt** gctagc ctga cctgag 5'

FIGURE 7

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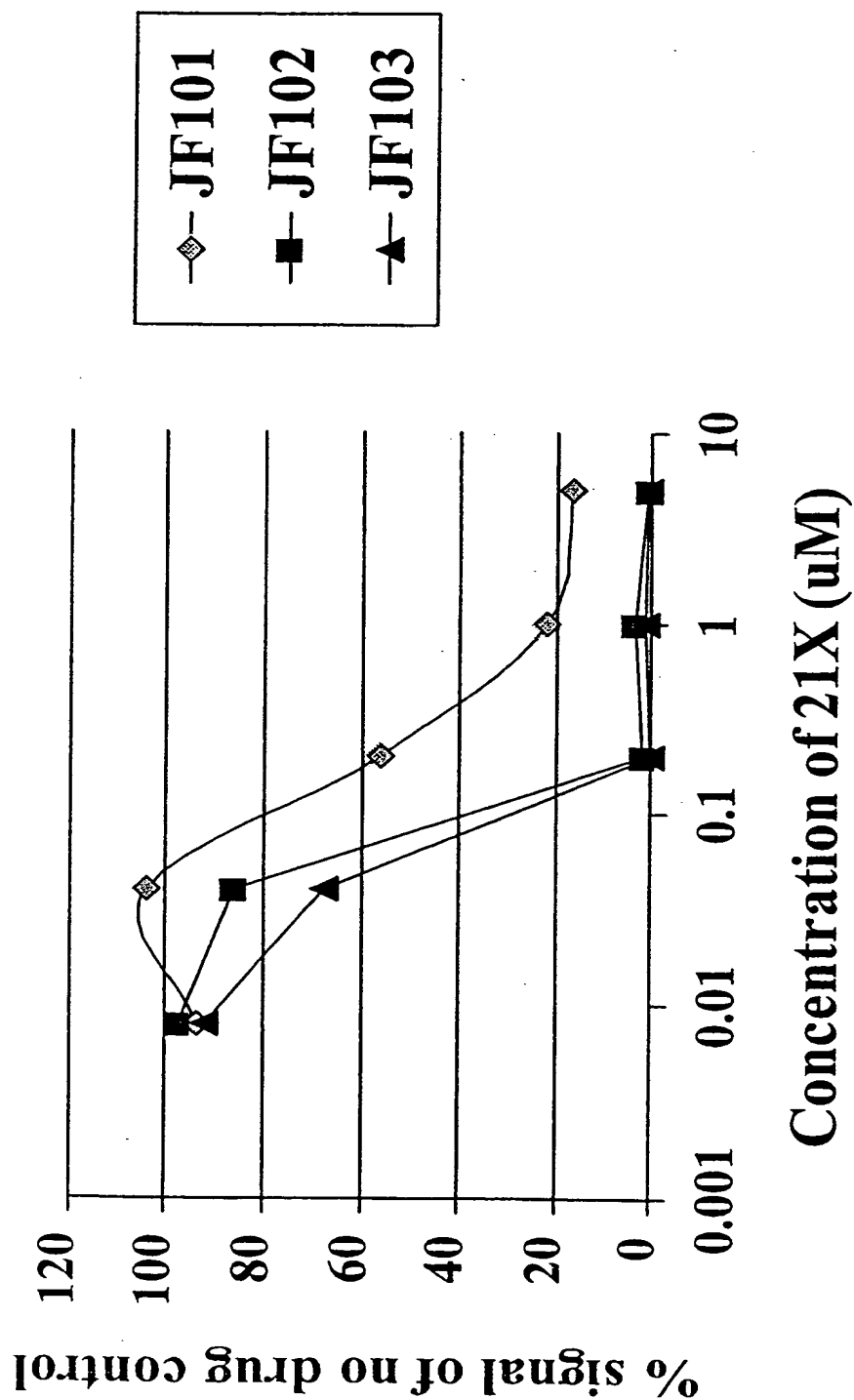


FIGURE 8A

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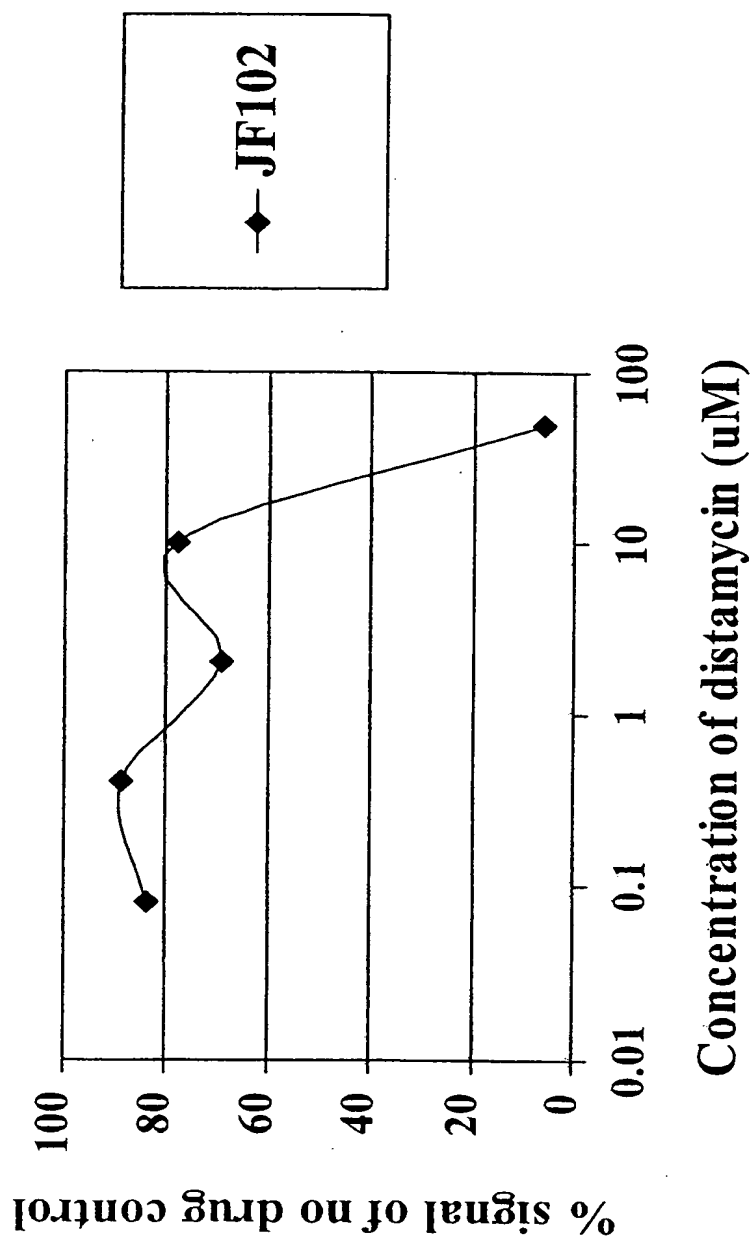


FIGURE 8B

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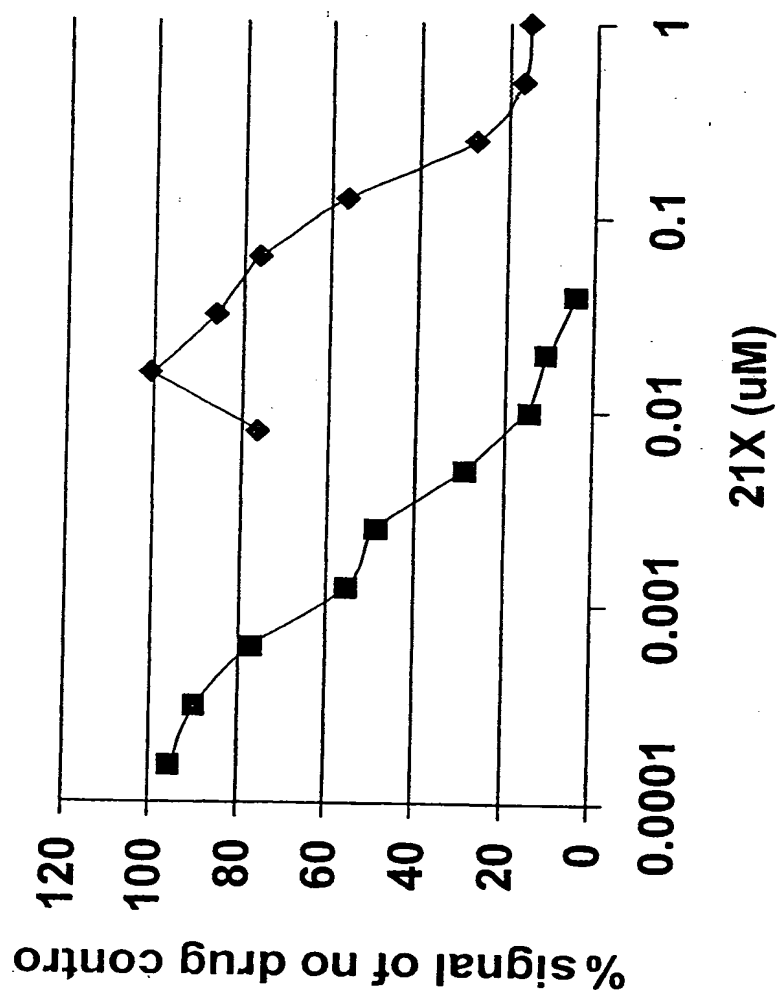


FIGURE 9

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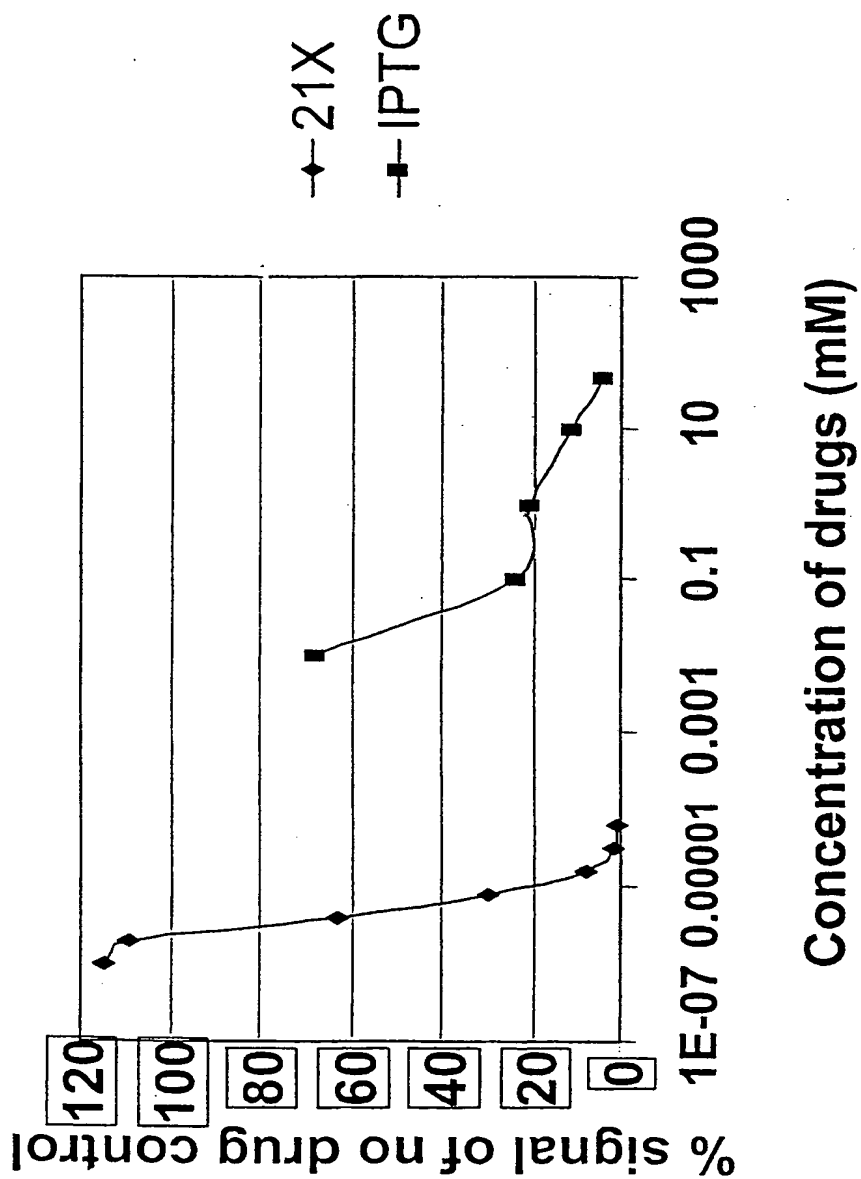


FIGURE 10

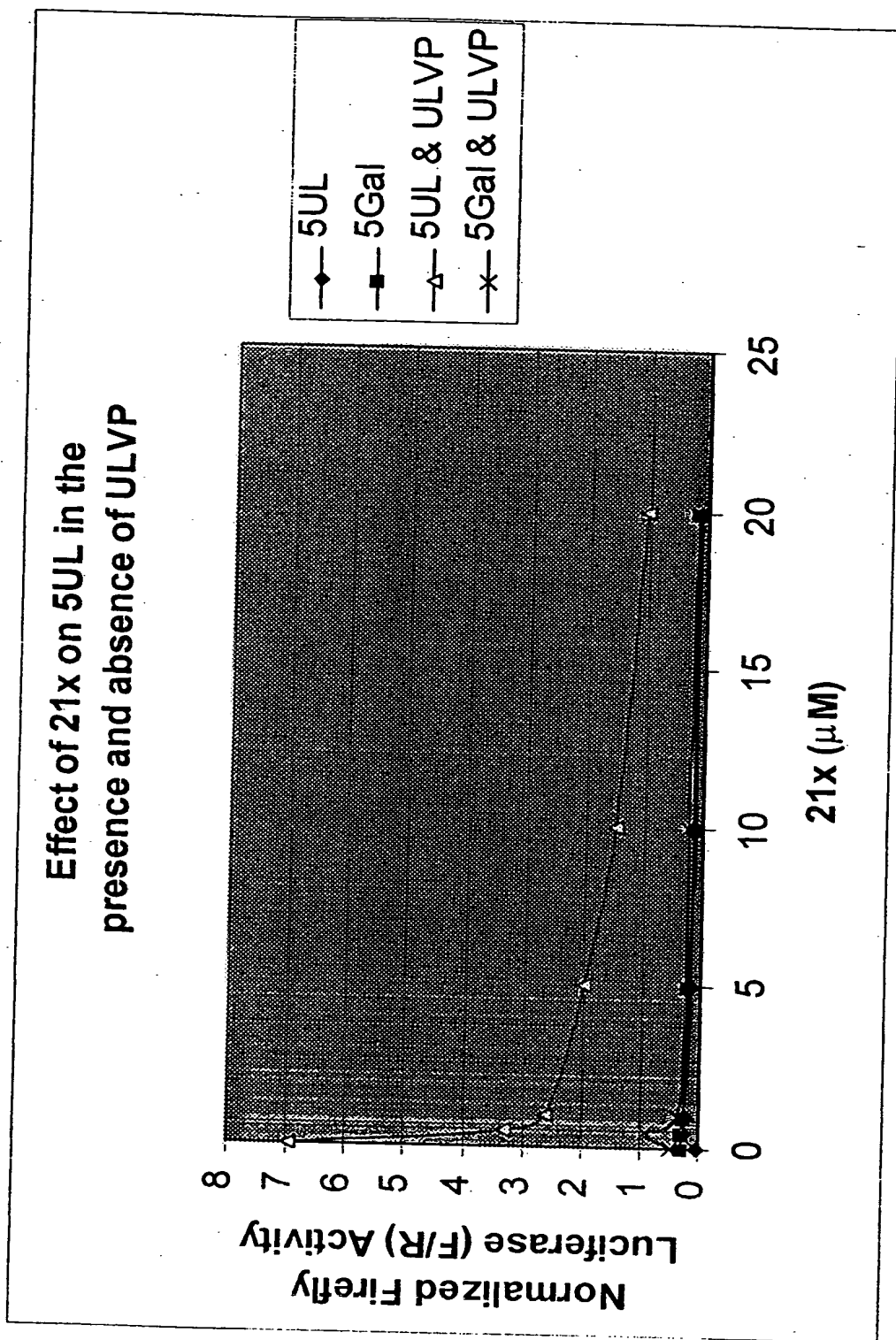


FIGURE 11

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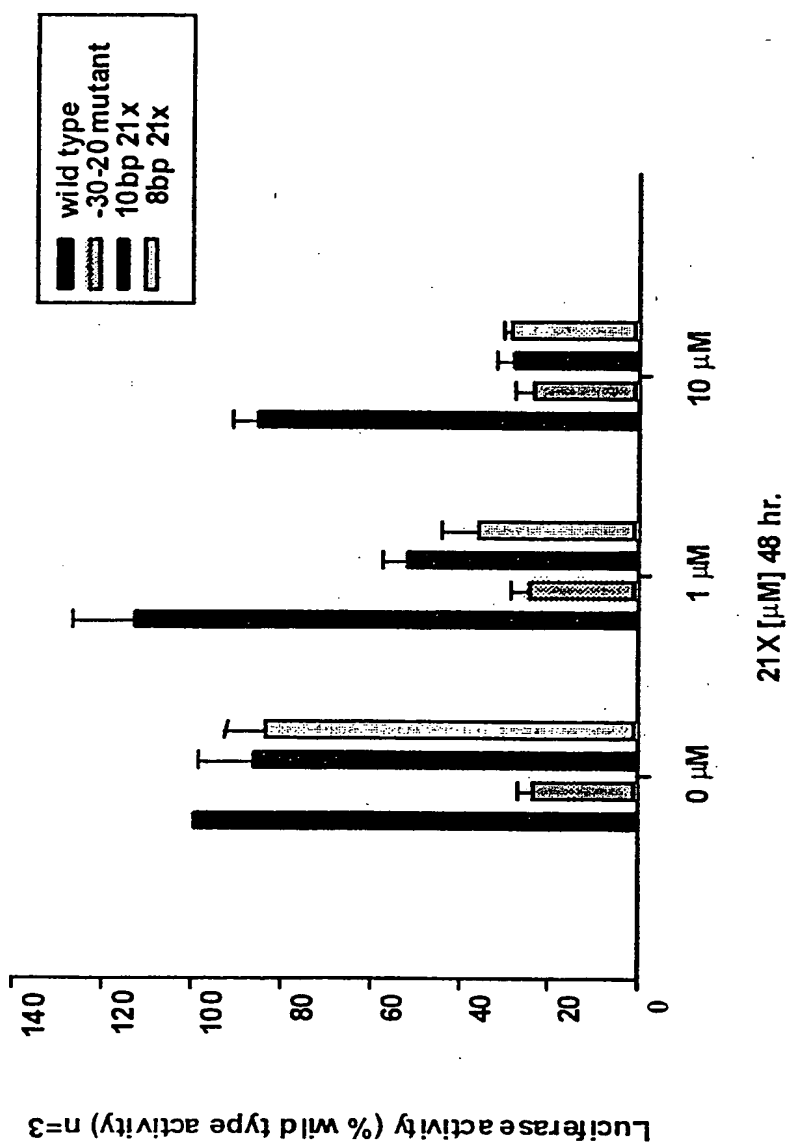


FIGURE 12

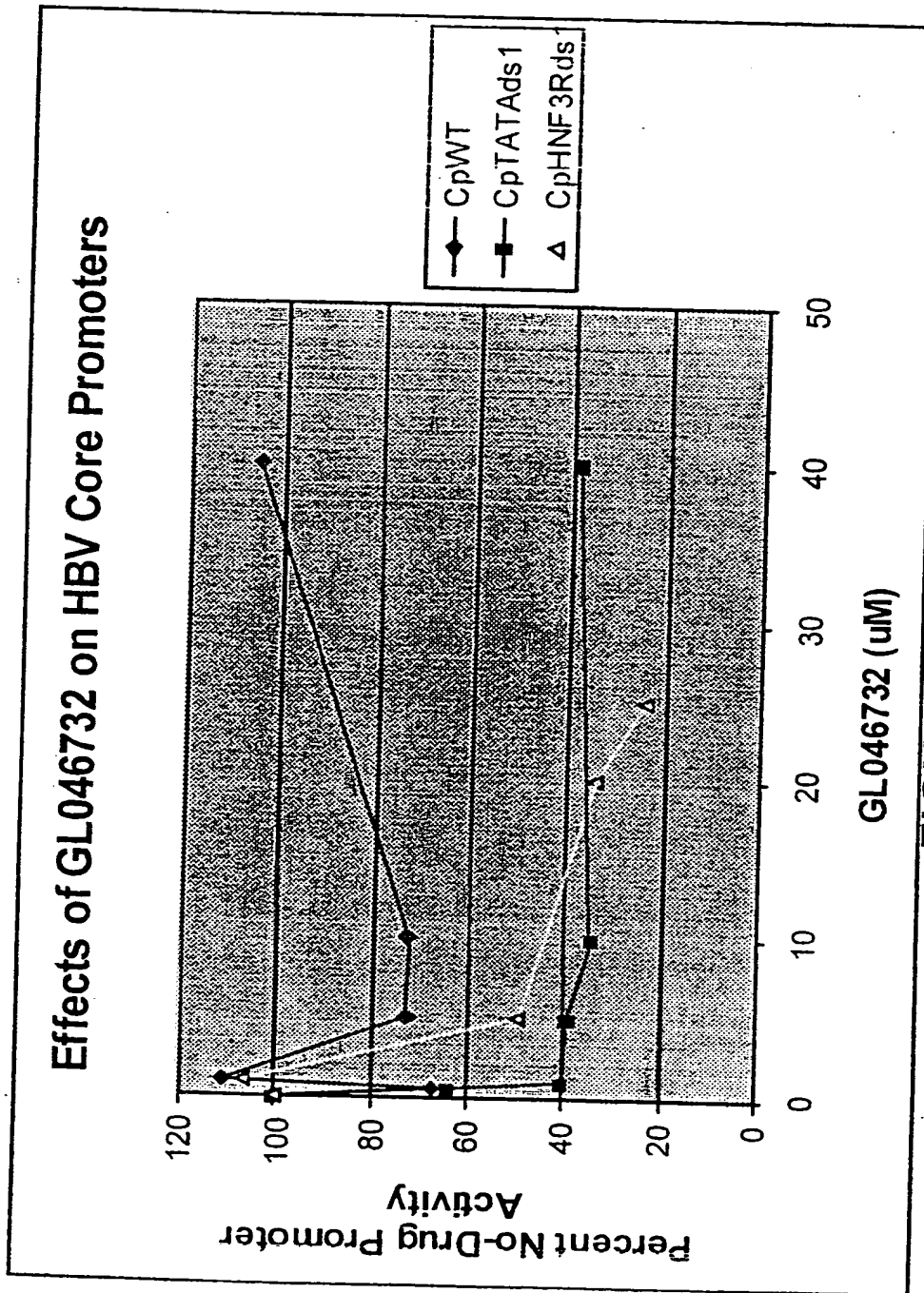


FIGURE 13

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TCAATATTGGCCATTAGCCATATTATTTCATTGGTTATATAGCATAAATCAATATTGGCTATTGGC
CATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCATGTCCAATATGACCG
CCATGTTGGCATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAG
CCCATATATGGAGTTCGCGTTACATAACTTACGGTAAATGGCCCGCTGGCTGACCGCCCAACG
ACCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCAT
TGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT
GCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCTGGCATTATGCCCAGTACA
TGACCTTACGGGACTTTCCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTG
ATGCGGTTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTACTCACGGGGATTTCGAAGTCT
CCACCCCATTTGACGTCAATGGGAGTTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTC
GTAACAACGCGATCGCCCGCCCCGTTGACGCAAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCT
ATATAAGCAGAGCTCGTTTTAGTGAACCGTCAGATCACTAGAAGCTTTATTGCGGTAGTTTATCAC
AGTTAAATTGCTAACGCAGTCAGTGCTTCTGACACAACAGTCTCGAACTTAAGCTGCAGTGACTC
TCTTAAGGTAGCCTTGCAGAAGTTGGTCGTGAGGCACTGGGCAGGTAAAGTATCAAGGTTACAAGA
CAGGTTTTAAGGAGACCAATAGAAACTGGGCTTGTGAGACAGAGAAGACTCTTGCGTTTTCTGATA
GGCACCTATTGGTCTTACTGACATCCACTTTGCTTTCTCTCCACAGGTGTCCACTCCCAGTTCA
ATTACAGCTCTTAAGGCTAGAGTACTTAATACGACTCACTATAGGCTAGCCAGCTTGAAGCAAGC
CTCCTGAAAGATGGAGGCGTCGCTGCCGGCCAGGCCGCCGAGACGGAGGAGTGGGCTTTTTCG
TCGAAAAATACCTCCGGTCCGATGTCGCGCCGCGGAAATTGTCGCGCTCATGCGCAACCTCAAC
AGCCTGATGGGACGCACGCGTTTTATTTACCTGGCGTTGCTGGAGGCTGTCTCEGCGTTCCTCAT
GGCCACCCGCAGCAGCGCCATATTTTCGGCGGATCTATGACCACTACGCCACGGGCGTCATCCCCA
CGATCAACGTCACCGGAGAGCTGGAGCTCGTGGCCCTGCCCCCACCCTGAACGTAACCCCGCTC
TGGGAGCTGTTGTGCCTGTGCAGCACCATGGCCGCGCGCCTGCATTGGGACTCGGCGGCCGGGGG
ATCTGGGAGGACCTTCGGCCCCGATGACGTGCTGGACCTACTGACCCCCCACTACGACCGCTACA
TGCAGCTGGTGTTCGAACTGGGCCACTGTAACGTAACCGACGGACTTCTGCTCTCGGAGGAAGCC
GTCAAGCGCGTCGCCGACGCCCTAAGCGGCTGTCCCCCGCGGGTCCGTTAGCGAGACGGACCA
CGCGGTGGCGCTGTTCAAGATAATCTGGGGCGAACTGTTTGGCGTGCAGATGGCCAAAAGCACGC
AGACGTTTTCCCGGGGCGGGGCGGCTTAAAAACCTCACCAAACAGACAATCGTGGGGTTGTTGGAC
GCCCACCACATCGACCACAGCGCCTGCCGGACCCACAGGCAGCTGTACGCCCTGCTTATGGCCCA
CAAGCGGGAGTTTGGGGGCGCGCTTCAAGCTACGCGTGCCCGCGTGGGGGCGCTGTTTGCACA
CGCACTCATCCAGCGCCAACCCCAACGCTGACATCATCCTGGAGGCGGCGCTGTGCGAGCTCCCC
ACCGAGGCTTGGCCCATGATGCAGGGGGCGGTGAACCTTTAGCACCTAATGAAGCTACTGTCTTC
TATCGAACAAGCATGCCCAAAAAGAAGAGAAAGGTAGATGAATTCCCGGGGATCTCGACGGCCC
CCCCGACCGATGTCAGCCTGGGGGACGAGCTCCACTTAGACGGCGAGGACGTGGCGATGGCGCAT
GCCGACGCGCTAGACGATTTGATCTGGACATGTTGGGGGACGGGGATTCCCCGGGTCCGGGATC
GCCAGGGATCCGTCGACTTGACGCGTTGATATCATCTAGAGCGGCCGAGGTACCTGAATAACTA
AGGCCGCTTCCCTTTAGTGAGGGTTAATGCTTTCGAGCAGACATGATAAGATACATTGATGAGTTT
GGACAAACCACAACCTAGAATGCAGTGAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGC
TTTATTTGTAACCATTTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGT
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CGCGGCGGGTGTGGTGGTTACGCGCACGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCC
TTTCGCTTTCTTCCCTTCTTCTCGCCACGTTGCGCGGCTTTCCCGTCAAGCTCTAAATCGGG
GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACCTTGATTAGGGT
GATGGTTCACGTAGTGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCAC
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TTGATTTATAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAA
TTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTTCGCTGTGTACCTTCTGAGGCGGAA
AGAACCAGCTGTGGAATGTGTGTGAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGA
AGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGC
AGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCCTAACTCCGC

FIGURE 14A

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CCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTT
ATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTT
TGGAGGCCTAGGCTTTTGCAAAAAGCTTGATTCTTCTGACACAACAGTCTCGAACTTAAGGCTAG
AGCCACCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT
TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCG
CAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGA
GGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCCTTGCGCAGCTGTGCTCGACGTTGTCA
CTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCAC
CTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCC
GGCTACCTGCCCATTGACACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAG
CCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTGTTC
GCCAGGCTCAAGGCGCGCATGCCCCAGGGCAGGATCTCGTCTGACCCATGGCGATGCCTGCTT
GCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCTGACTGTGGCCGGCTGGGTGTGG
CGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGG
GCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTGCGAGCGCATCGCCTTCTATCG
CCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAA
CCTGCCATCACGATGGCCGCAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTTTG
TGTGAAGATCCGCGTATGGTGCACCTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGC
CCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTAC
AGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTGAGAGGTTTTCACCGTCATCACCGAAACG
CGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTT
CTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAA
ATACATTCAAATATGTATCCGCTCATGAGACAATAACCCCTGATAAATGCTTCAATAATATTGAAA
AAGGAAGAGTATGAGTATTCAACATTTCCGTGTGCGCCCTTATTCCTTTTTTGCGGCATTTTGCC
TTCTGTTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGA
ACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACG
CCGGGCAAGAGCAACTCGGTGCGCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTACCA
GTACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCAT
GAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCCTT
TTTTGCACAACATGGGGGATCATGTAACCTGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCC
ATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATT
AACTGGCGAACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAG
TTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCC
GGTGAGCGTGGGTCTCGCGGTATCATTCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGT
AGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG
GTGCCTCACTGATTAAGCATTGGTAAGTGTGAGACCAAGTTTACTCATATATACTTTAGATTGAT
TTAAAACTTTCATTTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAA
AATCCCTTAACGTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTT
CTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG
GTGGTTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTTCAGCAGAGC
GCAGATACCAAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG
CACCGCTACATACTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG
TGTCTTACCGGGTTGGAATCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACGGG
GGGTTCTGTGCACACAGCCAGCTTGGAGCGAAGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGG
AGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGG
GTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGT
CGGGTTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGCGGAGCCTAT
GGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCTTGGCCTTTTGCTGGCCTTTTGCTCACATG
GCTCGACAGATCT

FIGURE 14B

FIGURE 15A

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CCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTTTATTTATGCA
GAGGCCGAGGCCGCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCT
AGGCTTTTGC AAAAGCTTGATTCTTCTGACACAACAGTCTCGAACTTAAGGCTAGAGCCACCAT
GATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTTCGGCTATG
ACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGC
CCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCG
GCTATCGTGGCTGGCCACGACGGGCGTTCCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGG
GAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCT
GCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG
CCCATTTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTG
TCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTTCGCCAGGCTC
AAGGCGCGCATGCCCGACGGCGAGGATCTCGTCTGACCCATGGCGATGCCTGCTTGCCGAATAT
CATGGTGGAAAATGGCCGCTTTTCTGGATTCTCGACTGTGGCCGGCTGGGTGTGGCGGACCGCT
ATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGC
TTCTCTGCTGCTTTACGGTATCGCCGCTCCCGATTTCGCAGCGCATCGCCTTCTATCGCCTTCTTGA
CGAGTTCTTCTGAGCGGGACTCTGGGGTTTGAAATGACCGACCAAGCGACGCCCAACCTGCCATC
ACGATGGCCGCAATAAAATATCTTTATTTTTCATTACATCTGTGTGTTGGTTTTTTGTGTGAAGAT
CCGCGTATGGTGCACCTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACC
CGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCT
GTGACCGTCTCCGGGAGCTGCATGTGTGAGAGTTTTACCGTCATCACCGAAACGCGCGAGACG
AAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTGATGATAATAATGGTTTCTTAGACGT
CAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCA
AATATGTATCCGCTCATGAGACAATAACCCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAG
TATGAGTATTCAACATTTCCGTGTGCGCCCTTATTCCTTTTTTTCGGCATTTTGCCTTCTGTTT
TTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
TACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTC
AATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAG
AGCAACTCGGTGCGCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTACCAGTCACAGAA
AAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAA
CACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACA
ACATGGGGGATCATGTAACCTGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATAACCAAC
GACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAGTGGCGA
ACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGAC
CACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTTATTGCTGATAAATCTGGAGCCGGTGAGCGT
GGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTA
CACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCAC
TGATTAAGCATTGGTAAGTGTGACACCAAGTTTACTCATATATACTTTAGATTGATTTAAACTT
CATTTTTTAATTTAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTA
ACGTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATC
CTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAAACAAAAAACACCGCTACCAGCGGTGGTTTTGT
TTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTTCAGCAGAGCGCAGATACC
AAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTA
CATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACC
GGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCCGGCTGAACGGGGGGTTTCGTG
CACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAG
AAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACA
GGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTGCGGTTTTCG
CCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGCGGAGCCTATGGAAAAACG
CCAGCAACGCGGCCCTTTTACGGTTCTTGGCCTTTTGCTGGCCTTTTGCTCACATGGCTCGACAG
ATCT

FIGURE 15B

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 September 2000 (08.09.2000)

PCT

(10) International Publication Number
WO 00/52179 A3

- (51) International Patent Classification⁷: C12N 15/63, 15/82, C12Q 1/68, C12N 5/10 94086 (US). TAM, Albert, W.; 1871 10th Avenue, San Francisco, CA 94122 (US).
- (21) International Application Number: PCT/US00/05728 (74) Agents: JUDGE, Linda, R. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).
- (22) International Filing Date: 3 March 2000 (03.03.2000) (81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/122,513 3 March 1999 (03.03.1999) US
60/154,605 17 September 1999 (17.09.1999) US
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant: GENELABS TECHNOLOGIES, INC.
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- (72) Inventors: LIM, Moon, Young; 10 Lido Circle, Redwood City, CA 94065 (US). EDWARDS, Cynthia, A.; 16965 Pine Way, Morgan Hill, CA 95037 (US). FRY, Kirk, E.; 2604 Ross Road, Palo Alto, CA 94303 (US). BRUICE, Thomas, W.; 6880 Shearwaters Drive, Carlsbad, CA 92009 (US). STARR, Douglas, B.; 1197 Bonita Avenue, Mountain View, CA 94040 (US). LAURANCE, Megan, E.; 1020 Shotwell Street, San Francisco, CA 94110 (US). KWOK, Yan; 995-1 Belmont Terrace, Sunnyvale, CA
- Published:
— With international search report.
- (88) Date of publication of the international search report:
21 December 2000
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DNA BINDING COMPOUND-MEDIATED MOLECULAR SWITCH SYSTEM

(57) Abstract: The present invention provides molecular switch system methods and compositions for use in regulatable gene expression. The system includes a nucleic acid construct which has a DNA response sequence for a transcriptional regulatory protein operably linked to a promoter, a compound binding sequence in the vicinity of the DNA response sequence, a transgene under the control of the promoter, and a DNA binding compound. In some cases, the molecular switch system further includes a nucleic acid sequence encoding a transcriptional regulatory protein operably linked to a second promoter. The invention further provides a method for screening compounds for the ability to function in the molecular switch system and thereby regulate gene expression.

WO 00/52179 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/05728

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/63 C12N15/82 C12Q1/68 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 97 10337 A (BAYLOR COLLEGE MEDICINE) 20 March 1997 (1997-03-20)</p> <p>page 5, line 10 -page 6, line 4 page 7, line 1-26 page 15, line 1 -page 20, line 28 page 62, line 1 -page 63, line 8; examples 1,2,5-8</p> <p style="text-align: center;">--- -/-</p>	<p>1,4, 9-13, 16-18, 20-24, 31-33</p>

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

14 September 2000

Date of mailing of the international search report

26/09/2000

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/05728

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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P,X	<p>WO 99 25856 A (HEDDLE JOHN A ;SWIGER ROY R (CA)) 27 May 1999 (1999-05-27)</p> <p>page 2, line 16 -page 3, line 32 page 6, line 11 -page 9, line 25 page 12, line 19 -page 15, line 31</p>	1,2,4, 6-8,11, 12,16, 18-22, 24,25, 31-33
T	<p>CHEN I G ET AL: "Groucho/TLE family proteins and transcriptional repression" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 249, no. 1-2, May 2000 (2000-05), pages 1-16, XP000946056 ISSN: 0378-1119 the whole document</p>	25,26

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